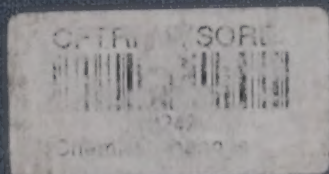


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Chemical Changes in Food during Processing



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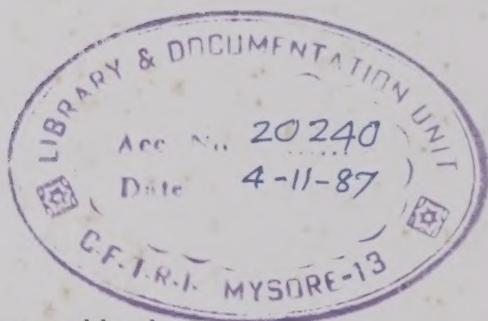
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Preface

This volume results from the Eighth Basic Symposium held by the Institute of Food Technologists in Anaheim, California on June 8–9, 1984. The theme of the symposium was “Chemical Changes in Food during Processing.”

The speakers included a mix of individuals from academic institutions, governmental agencies, and the food industry. Twenty speakers discussed topics ranging from the basic chemistry relating to food constituents to the more applied aspects of chemical changes in food components during food processing.

It was the intent of the organizers to bring together a group of speakers who could address the chemistry of changes in food components during processing from a mechanistic point of view. As a consequence, the proceedings of this symposium emphasize the basic chemistry of changes in food constituents from a generic perspective which is intended to provide the reader with a background to address more specific problems that may arise.

The book is introduced with an overview of the major chemical changes in food during processing to orient the reader as to its scope and to give a general feel for the areas covered. A major part of the initial section of the volume focuses on the chemistry and biochemistry of oxygen and oxidative reactions as influenced by environmental factors, metals, enzymes, and ionizing radiation. These pervasive reactions are major causes for changes observed in foods during processing and storage. Recent emphasis given to the so-called “active-oxygen species” in initiating oxidative alterations in biological materials dictates a treatment of their relevance to changes in foods. Although we are accustomed to thinking of metals primarily in terms of redox reactions, a variety of ionic reactions catalyzed by these agents and their chelates is discussed from a mechanistic point of view.

There is a natural progression to free radical chemistry and alterations induced in lipids from autoxidation, from thermally induced

changes, from ionizing radiation, and from enzymatic modifications. Enzymatic oxygenation of lipids in plants may play a key role in their physiology analogous to the lipid oxidation cascade systems in animals. A major concern of the food scientist is to minimize damage from oxidative reactions in foods, and a basis is given for a better understanding of antioxygenesis at the molecular level.

Two groups of enzymes that are of principal importance to the food scientist are discussed in some detail. The oxidoreductases provide a basis for establishing blanching limits for fruits and vegetables. On the other hand, they can serve as focal points for undesirable changes in foods, as in enzymatic browning. The mechanistic discussions of selected oxidoreductases will give the reader a fundamental grasp of the diversity in how these enzymes effect change. The hydrolases are ubiquitous enzymes designed to use water for breaking down substrates in foods. However, an understanding of the mechanisms of hydrolases can provide the food scientist with opportunities for controlling or even diverting the course of their reactions.

Changes in biopolymers during processing can alter their physical and nutritional properties. The reader is treated to a comprehensive discussion of textural alterations in foods as affected by chemical, physical, and enzymatic changes in pectins, starches, and cellulose. Proteins possess a large number of side-chain functional groups subject to chemical alterations. A discussion of the reactivities of amino acid residues in proteins paves the way for a comprehensive discussion on the effects of environmental elements, such as heat, light, pH, and oxygen, on the amino acid residues of proteins. An important reaction affecting not only the nutritive quality of proteins but also the color and flavor of foods is nonenzymatic browning. Some new aspects relating to the polymeric products of the Maillard reaction are discussed.

Throughout this volume it is shown that the effects of environmental factors are constantly impacting on the quality of food constituents. This is especially true in the destruction of vitamins during food processing. A thorough discussion relates many environmental factors to rates of vitamin destruction. In addition to the kinetics of vitamin destruction under defined conditions, the organic reaction pathways involved in vitamin loss are detailed.

The organoleptic properties of foods cannot be underestimated. The aromas, taste, and colors of foods serve as a basis for the selection and palatability of foods which, in turn, contributes to consumer nutrition. The origins and changes in food flavors are complex, but are treated in this volume in a very thorough fashion from a sound chemical viewpoint. Likewise food colorants are represented by substances

as diverse as the lipophilic carotenoids to the water-soluble anthocyanins. The chapter on origins of colors as well as their alterations during food processing brings the reader abreast of current knowledge on chemical changes in the major natural food pigments.

As noted previously, the major environmental factors that impact on the quality of foods are well known. However, quantitative treatments of the complex interactions between food constituents and their environment are difficult and often require simplifying assumptions for adequate modeling.

The final chapter in the book includes a discussion of the effects of water activity, light intensity, oxygen concentration, and temperature on lipid oxidation, nonenzymatic browning, enzymatic reactions, and protein-lipid interactions. General models of environmental effects on food deterioration are illustrated by a case of optimization of dehydration to maximize nutrient retention in a dehydrated food.

This volume represents a broad treatment of chemical changes in food processing. The major components of foods are discussed without a commodity orientation but within the context of functional group chemistry of the functional constituents. It is hoped that thoughtful readers will find it of value in some aspect of their professional activities.

The success of the Eighth Basic Symposium was the result of the expert assistance of the members of the 1984 Basic Symposium Committee: Dr. Bernard J. Liska, 1984 President of IFT; Calvert L. Willey, Executive Director of IFT; John B. Klis, Director of Publications; and the IFT staff who provided support, publicity, and coordinated physical planning and details that make such a two-day symposium a success.

For the eighth time, John Klis coordinated all details of interface with the publisher, and Anna May Schenck, JFS Assistant Scientific Editor, served as copy editor for the proceedings. Their capabilities, professionalism, and patience in the face of pending deadlines have brought these proceedings to fruition.

However, it is to the authors of the chapters of this book that we owe our deepest gratitude. Without their expertise, persistence, and cooperation, this symposium and book would not have been possible.

THOMAS RICHARDSON
J.W. FINLEY

Chemical Changes in Food during Processing— An Overview

*O. Fennema*¹

FOOD PROCESSING IN PERSPECTIVE

Without exception, the major methods currently used to commercially preserve foods, namely, heating, cooling (chilling, freezing), reduction of water activity (concentration, dehydration), and fermentation, were originally developed with little knowledge of the chemical consequences. However, since their commercialization, science has caught up with practice not only for these well-established processes but also for more recent processing techniques (e.g., alkali processing of soy proteins). As a consequence, a considerable body of information has accumulated concerning the chemical consequences of food processing on the sensory properties, nutritive value, and wholesomeness of foods. It is indisputable that processing can and does result in extensive chemical changes in food, with the kind and degree of these changes depending upon the food, the specific process, and the associated handling and storage procedures employed.

In order to gain a proper perspective about chemical changes that occur in food during processing, it is useful to first consider the ad-

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vantages and disadvantages of commercial food processing and then to discuss the major kinds of chemical reactions encountered during processing and their consequences on the sensory properties, nutritive value, and wholesomeness of foods

Food processing can result in several advantages, some of which are substantial:

- Lessened hazards from microbial pathogens
- Lessened spoilage (microbial, enzymatic)
- Inactivation of heat-labile, antinutritional substances (e.g., trypsin inhibitors in soy proteins)
- Year-round availability of seasonal foods
- Availability of perishable foods (in preserved forms) in regions far removed from the site of production
- Increased convenience
- Increased variety of foods, some with enhanced sensory properties (bakery and confectionery products, meat analogs, fermented foods)

Food processing can also result in disadvantages, and these should be acknowledged and the causes studied so that remedial measures can be devised. The major disadvantages are (1) harm to the sensory properties and nutritive value of some foods, particularly when severe methods of processing (heat sterilization, air drying) are applied to tissue foods, and (2) development during some kinds of processing and handling of new chemicals that must be classed as toxicologically undesirable. Damage to nutritional value and development of toxicologically undesirable compounds during processing deserve further comment.

Critics of processed foods often cite differences in the nutritive value of canned and garden-fresh foods. This is an improper comparison. Harm to the nutritive value of processed foods can be fairly assessed only if (1) the nutritive value of a ready-to-eat processed food is compared to that of a commercially fresh food ("fresh" as purchased in a retail outlet) that has been brought to a ready-to-eat state. It is unfair and unrealistic to compare ready-to-eat processed foods with garden-fresh raw foods, since the latter are seldom available, and even when they are, they are frequently cooked (nutrient loss) to produce a ready-to-eat condition. (2) It is recognized that the importance of a decrease in a given nutrient depends upon the kind of nutrient (whether it is abundant or meager in the average diet), the kind of food (does this food supply a major or a minor amount of the nutrient in the total diet?), and the condition of the consumer (healthy or ill, old or young, malnourished or well fed, active or sedentary, etc.).

With regard to toxic substances, it is true that some develop in foods during certain kinds of processing. However, these undesirable constituents exist at very low levels, and there is no indication that they have a significant negative impact on the health of normal persons who consume properly processed foods. This statement is, no doubt, less than satisfying to critics of processed foods. To support their negative position they are sometimes able to cite adverse effects in animals achieved when a chemical that is added to or develops in food during processing is injected in or is fed to animals at a very high concentration in the diet. In judging such results, one needs to keep in mind that the use of animals for assessing the safety of foods or food constituents, although clearly justifiable and appropriate, produces results that entail at least three formidable barriers to accuracy: (1) assessment of sublethal, adverse effects in animals is an inexact science; (2) responses of various animal species to a given kind and dose of a chemical can vary substantially; and (3) animal data can be extrapolated to humans only with considerable uncertainty, even under the best of circumstances. This uncertainty increases when a test substance is fed to animals at levels far in excess of those normal encountered in human diets. It is believed possible that massive doses in animals may produce "metabolic overload" with the formation of metabolites that would not occur at lower doses (Committee on Food Protection 1980). Interactions among dietary components may also increase the uncertainty of extrapolation. Interactions may lessen or intensify the effect of the given test substance, and the response direction is not always known. Even when known, it is difficult to quantify.

With the foregoing points in mind, it is reasonable and proper that processed foods should occupy a prominent place in the diets of humans. Although this statement is, in my view, incontestable, a word of caution is in order. In developed countries of the world, food processed to one degree or another represents well over half of a typical diet, and this percentage is increasing. Thus, it is clear that processed foods, especially those that are major components of the diet, must in the future be nutritionally more complete.

CHEMICAL CHANGES DURING PROCESSING

Chemical changes that occur during food processing are numerous, and they can be desirable, undesirable, of questionable consequence, or a combination thereof. Attention here will be given mainly to those changes that can be categorized as desirable or undesirable, and only a few illustrative instances will be cited in each subcategory.

Desirable Changes during Processing

Many desirable changes occur in foods during processing, and these changes can influence sensory properties, functional properties, and/or nutritive value.

Development or Preservation of Pleasing Colors and Flavors. Desirable colors and flavors can develop during the processing of food tissues, such as meats, coffee beans, nuts, and olives, and during the processing of fabricated foods, such as bakery products, confectionery products, snack foods, and breakfast cereals. Desirable flavors develop during the fermentation of foods, such as cheese and alcoholic beverages, during the postharvest ripening of fruits, and during the disruption of plant tissues. Preservation of color and flavor is often achieved by the addition of chemicals, such as antioxidants, or the removal of undesirable components, such as glucose from egg white to retard browning of the dried product.

Numerous, complex chemical reactions are involved in the aforementioned events, with the Maillard reaction, Strecker degradation, caramelization, oxidation of lipids, and reactions catalyzed by endogenous enzymes playing predominant roles.

Improvement or Preservation of Texture. Examples of desirable modification of texture include the softening of plant tissues by heat; the firming of plant tissues through the action of calcium and endogenous pectin methylesterase (the later being intentionally decompartmentalized and activated by a mild heat treatment); tenderization of meat by the addition of proteases; development of a desirable texture in meat analogs; gelling, coagulation, or firming of egg products, puddings, and bakery products by heat; and formation of cheese by the development (fermentation) or addition of acid to milk. Texture degradation of some plant tissues can be retarded by blanching to inactivate enzymes.

Hydrolytic reactions, many of which are enzyme catalyzed, appear predominantly among reactions that cause softening of texture. Associative reactions, depending on hydrogen bonding, hydrophobic association, and cross-linking of polymers mediated by multivalent ions, are important causes of texture firming.

Improvement of the Functionality of Food Ingredients. Examples of improved functionality by processing include heat denaturation of whey proteins in dried milk intended for bread making, prerigor freezing of meat intended for sausage making (to enhance its water-

holding properties), alteration in the functionality of starch by gelatinization or chemical modification, alkali processing of soy proteins to impart new textural properties, control of thiol-disulfide interchange reactions in gluten to develop proper rheological properties in bread doughs, and increasing the sweetness of corn syrups by isomerizing glucose to fructose.

Inactivation or Control of Enzymes. It is often desirable to inactivate endogenous enzymes, for example, lipases, lipoxygenases, proteases, phenolases, amylases, and ascorbic acid oxidase, in food tissues and biological fluids. If this is not done in fruits and vegetables prior to freezing or drying, undersirable enzyme-catalyzed reactions will occur that damage color, flavor, texture, and nutritive value of foods during long-term storage. Heat is the most common means of enzyme inactivation, but other methods such as pH control, sequestering of metals, and chemical inhibition (e.g., SO_2 to inhibit phenolase) are also employed. Reactant removal (e.g., O_2) or reactant modification (e.g., reduction of quinones by ascorbic acid or methylation of quinones with catechol *O*-methyltransferase in the presence of a methyl donor such as *S*-adenosylmethionine iodide to prevent undesirable phenolase-catalyzed oxidative browning of quinones in plant tissues) also can be used to control enzyme activity (Finkle and Masai 1964; Finkle and Nelson 1963).

Inactivation of Antinutritional Substances and Other Approaches to Improving Nutritional Value. Naturally occurring antinutritional substances occur in some foods. Examples are trypsin inhibitors and hemagglutinins in legumes, thiaminase in fish, avidin in eggs, and phytates in cereal grains. Many antinutritional substances are proteinaceous and therefore are subject to inactivation during moderate heating of moist foods (Chichester and Lee 1981; Conn 1981; Whitaker, 1981). In other instances removal or chemical modification is a feasible approach. Examples involve enzymatic hydrolysis (or removal) of lactose from milk that is intended for lactose-intolerant individuals, the reduction of stachyose and raffinose (flatulence-producing chemicals) concentrations in leguminous seeds by germination, and the removal of phytates from grain by milling (Committee on Food Protection 1973; Nakayama 1981).

In some instances, moderate heat processes will result in improved nutritive value of foods. This can occur, for example, with cereals where bound nicotinic acid can be partially freed by heating (Clegg 1963; Koetz and Neukom 1977; Rajalakshmi *et al.* 1964) and with starch

granules and some proteins where heat will enhance the rapidity and/or completeness of digestion (Cheftel *et al.* 1985).

The intentional addition of nutrients (minerals, amino acids, vitamins) is also sometimes practiced, and this can result in nutritive value that equals or exceeds that of the unprocessed food (Borenstein 1979; Committee on Chemistry and Public Affairs 1980; Committee on Food Protection 1975).

Undesirable or Potentially Undesirable Changes during Processing

Many undesirable or potentially undesirable chemical changes can occur in foods during processing, and these changes can influence sensory properties, functional properties, nutritive value, and toxicity.

Damage to Color and Flavor. Damage of this kind is common and examples include the development of cooked flavors in milk during heat sterilization and degradation of the color of green vegetables during heat sterilization or air drying.

Damage to Texture during Processing. Common examples include excessive softening of fruits and vegetables during thermal processing, toughening of fish muscle during frozen storage, firming of bread during storage at refrigerator temperatures, emulsion destabilization by heating or freezing, coagulation of sterilized milk during storage, cold shortening of red meats when excessively cooled while in a pre-rigor state, and adverse textural changes in tissue foods during air drying.

Damage to Functional Properties of Food Ingredients during Processing. Some examples are impairment of the foaming properties of egg white by heat, a reduction in protein water-holding capacity or solubility by heat, and development of undesirable foaming in fat during extended deep-fat frying.

Damage to the Nutritional Properties and/or the Development of Toxic or Potentially Toxic Constituents during Processing. This aspect is best approached by considering the important classes of food constituents.

Vitamins. Some of the vitamins are labile and therefore losses are incurred during certain kinds of food processing (Bender 1978; Harris and Karmas 1975; Tannenbaum *et al.* 1985). Vitamins C, D, E, A, and folate are especially prone to inactivation by oxidation, and vi-

tamins C, folate, thiamin, and B₆ are subject to degradation during heating in the presence of water. Riboflavin is especially susceptible to light-catalyzed degradation.

Proteins. The nutritive value and sometimes wholesomeness of proteins can be modified by heating, oxidation, exposure to alkaline conditions, and by reactions with other organic molecules. Extensive information on these reactions has accumulated in recent years (Cheftel *et al.* 1985; Finot 1982; Gould and MacGregor 1977; Hurrell and Carpenter 1977; Feeney 1980; Lea and Hannan 1949; Lee *et al.* 1975; Masters and Friedman 1980; Mauron 1977; Neukom 1980; Satterlee and Chang 1982; Waller and Feather 1983; Yannai 1980).

Hearing of moist, acidic proteins in the absence of both oxygen and active carbonyl groups can result in unfolding of the molecule (denaturation), some cross-linking (isopeptide bonds), and, if the heating is severe, some destruction of component amino acids (Hurrell and Carpenter 1977; Mauron 1977). Denaturation usually results in inactivation of enzymes and proteinaceous antinutritional substances (Chichester and Lee 1981; Schwimmer 1981; Whitaker 1981). Heat treatments administered under these conditions and in accord with good manufacturing practices usually have either positive or negligible negative effects on protein nutritive value.

Heating of moist proteins in the presence of active carbonyl groups (e.g., reducing sugars and products of lipid oxidation) will favor the Maillard reaction and Strecker degradation (when dicarbonyls are present). These reactions will not only cause significant changes in sensory properties, but will also have a negative effect on nutritive value. Whether toxic substances develop to a level of significance during browning is still a matter of investigation and debate (Feeney and Whitaker 1982; Finot 1982; Hurrell and Carpenter 1977; Lee *et al.* 1975; Satterlee and Chang 1982; Waller and Feather 1983; Yannai 1980).

Heating of moist proteins in the presence of alkali results in two important changes that influence nutritive value: racemization and formation of compounds of the lysinoalanine type (Feeney 1980; Gould and MacGregor 1977; Masters and Friedman 1980; Mauron 1977; Satterlee and Chang 1982). Both occurrences have negative effects on protein nutritive value, but appear to have no toxicological effects of significance.

Oxidation of proteins whether or not accompanied by heat will result in thiol-disulfide interchange reactions, cross-linking (e.g., S—S, dityrosine), and the formation of degradation products. Among these, the dityrosine linkages and the oxidative degradation products are known to detract from protein nutritive value (Feeney 1980; Finot

1982; Mauron 1977; Neukom 1980; Satterlee and Chang 1982). Toxic constituents can form during protein oxidation, but it seems likely that the kinds and concentrations are insufficient under mild oxidizing conditions to pose a risk to health.

Proteins can also interact with aldehydes and sugars to form cross-links and with lipids to form complexes. These reactions can affect food texture and protein nutritive value, the later consequence usually being of minor importance (Cheftel 1979; Cheftel *et al.* 1985; Shenouda and Pigott 1977).

Major reactions that proteins can undergo during processing and handling of foods are summarized in Fig. 1.1.

Lipids. Lipids, especially when unsaturated, undergo many kinds of chemical changes during processing, and some of these changes can affect their nutritional value and wholesomeness (Artman and Smith 1972; Bolland and Koch 1945; Carpenter and Slover 1973; Firestone *et al.* 1961; Matsuo 1962; Morton 1977; Nawar 1985; Richardson, 1984; Yannai 1980). Unsaturated lipids are susceptible to oxidation when exposed to oxygen, radiant energy, and/or a variety of organic and inorganic catalysts, and when this occurs, several chemical changes can be observed that are of importance nutritionally and perhaps toxicologically:

1. *Formation of hydroperoxides.* These are more toxic than the unoxidized parent compounds and some believe them to be carcinogenic (Cutler and Schneider 1973).

2. *Partial conjugation of the double-bond system in lipids.* Conjugated fatty acids apparently are more likely to convert to the trans configuration and are also more likely to engage in dimerization and polymerization reactions than the nonconjugated cis counterparts. Fatty acid dimers and polymers when fed to rats have no nutritive value and cause growth suppression and poor reproduction (Matsuo 1962; Perkins 1967; Yannai 1980).

3. *Partial conversion of natural cis fatty acids to trans fatty acids.* Trans essential fatty acids are devoid of physiological functionality and are much more susceptible to polymerization during heating than the cis counterparts. When present in the diet of animals at high concentrations for long periods, trans fatty acids may have effects that are unhealthful (Carpenter and Slover 1973; Kummerow 1974; Matsuo 1962; Perkins 1967; Sgoutas and Kummerow 1970; Sommerfeld 1983).

Heating can also cause undesirable changes in lipids, the formation of cyclic fatty acid monomers being of particular concern (Artman and

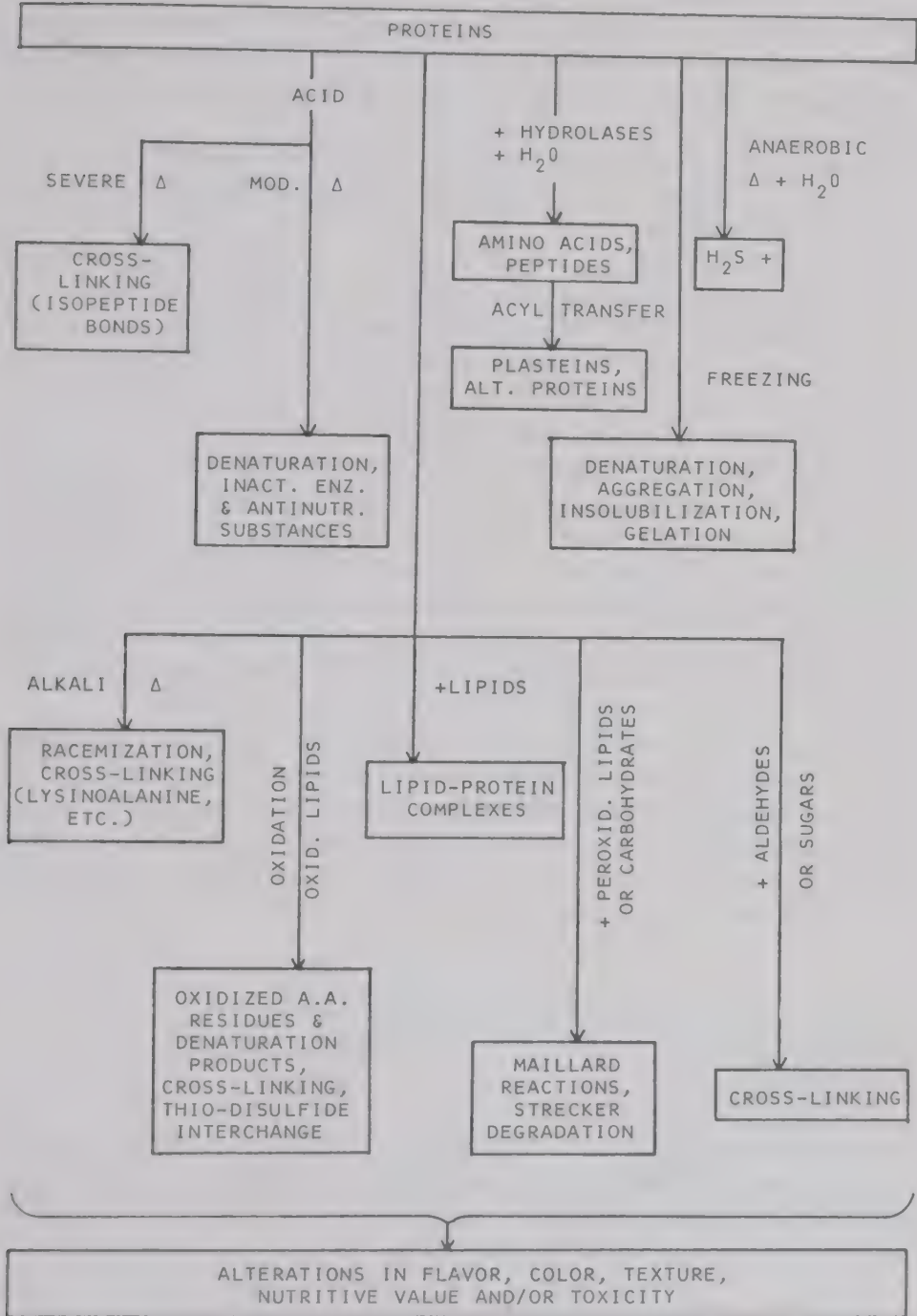


FIG. 1.1. Major chemical reactions that proteins can undergo during the processing and handling of foods.

Smith 1972; Firestone *et al.* 1961; Yannai 1980). Artman and Smith (1972) concluded that cyclic fatty acids are probably the most toxic products that develop during the heating of fats in the presence of air.

Peroxidizing lipids also exert negative effects on the nutritive value and perhaps wholesomeness of foods by their chemical interaction with proteins and vitamins.

Major chemical reactions that lipids can undergo during the processing and handling of foods are summarized in Fig. 1.2

Carbohydrates. Carbohydrates are generally less susceptible than proteins and lipids to processing-induced chemical changes that impair nutritive value or increase toxicity. Carbohydrates with active carbonyl groups do, of course, participate readily in Maillard reactions and Strecker degradation of proteins and thereby have an adverse effect on protein nutritive value and perhaps toxicity. Other types of carbohydrate reactions that would have a negative effect on nutritive value and perhaps wholesomeness of foods are caramelization of sugars and thermal degradation of carbohydrates in an aqueous environment (e.g., formation of furfural, hydroxymethylfurfural) (Houminer 1973; Lee *et al.* 1975; Rice 1972; Simonyan 1969; Birch 1977; Hodge and Osman 1976; Whistler and Daniel 1985).

Major chemical reactions that carbohydrates can undergo during the processing and handling of foods are summarized in Fig. 1.3.

Other Components. Processing and handling also results in the intentional addition of chemicals to foods and, on occasion, contamination of foods with unwanted chemicals. These sources of chemicals need to be continually monitored with care.

MEANS OF CONTROLLING CHEMICAL REACTIONS IN FOODS DURING PROCESSING AND HANDLING

Processors and handlers of food have at their disposal a variety of means to control the kinds and rates of chemical reactions that occur in foods, and these means are summarized in Table 1.1.

CONCLUSION

Much remains to be learned about chemical reactions that occur during the processing of foods and the means for exerting effective control over these reactions. This statement applies equally well to

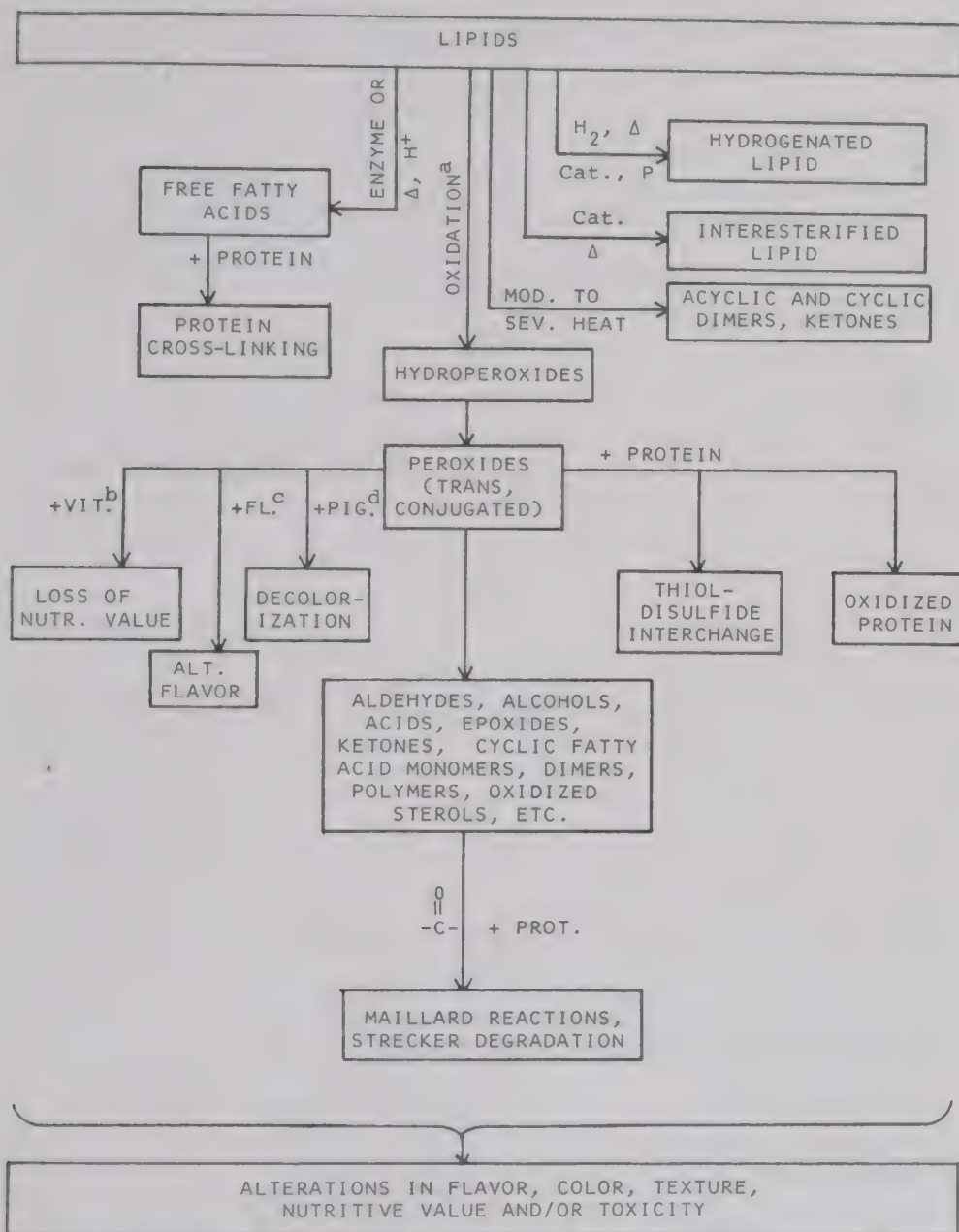


FIG. 1.2. Major chemical reactions that lipids can undergo during the processing and handling of foods.

^aOxidation by enzymes, metals, myoglobin, chlorophyll, and/or irradiation.

^bVIT., vitamins A, C, D, E, and folate.

^cFL., flavor; ^dPIG., carotenoids, chlorophyll, myoglobin, and anthocyanins.

TABLE 1.1 FACTORS GOVERNING THE KINDS AND RATES OF CHEMICAL REACTIONS THAT OCCUR IN FOODS

1. Product composition
Chemical properties of constituents
pH, a_w , catalysts, $[O_2]$
2. Process and storage variables
Time and temperature
Composition of the atmosphere (e.g., control atmosphere storage packaging)
Chemical or biological treatments
Protection from light, contamination, and physical damage

chemical reactions that influence sensory properties and to chemical reactions that influence nutritive value or wholesomeness of foods. Nonetheless it seems abundantly clear that foods processed and handled in accord with good manufacturing practices are valuable, wholesome components of the diet.

ACKNOWLEDGMENT

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Chemistry of Reactive Oxygen Species

*Christopher S. Foote*¹

INTRODUCTION

Oxidation is one of the most significant causes of foodstuff deterioration. It can lead to rancidity, off-flavor and -color, deterioration of texture, and other damage. Toxic materials may also result from the oxidation of naturally occurring substances (Pryor 1984). For example, cholesterol epoxides and hydroperoxides are thought to be carcinogenic or mutagenic (Koch and Schenck 1967; Sporer *et al.* 1982); they are among the products of oxidation of cholesterol (Smith *et al.* 1979).

In addition to the damage to food, substances may be present in food which cause oxidative damage to organisms that consume it: For example, the herbicide Paraquat owes its toxicity to oxidative chemistry (Rabinowitch and Fridovich, 1983). In addition, there are photochemically active compounds known as "photodynamic" sensitizers present in plants (probably for defense) which cause photooxidative damage (e.g., skin lesions) to organisms that feed on the plants (Arnason *et al.* 1983).

There are also natural agents that protect against oxidative damage, both in the foodstuff itself and in its ultimate consumer. For ex-

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ample, antioxidants such as tocopherol (vitamin E), β -carotene, ascorbic acid, and thiols such as cysteine are powerful inhibitors of many kinds of oxidation. Metal chelators can also play an important role in inhibiting oxidation processes in which metals play a catalytic role. Enzymes such as superoxide dismutase, catalase, and glutathione peroxidase protect against damaging reactive species such as superoxide ion, H_2O_2 , and organic hydroperoxides, respectively.

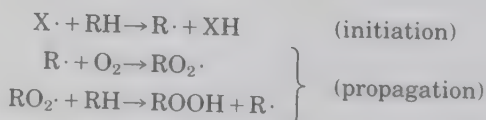
In order to make sense of the many oxidation pathways and, ultimately, to gain control over them, it is important to have an understanding of the various reactive species and reaction mechanisms which occur under various conditions. Various processes can be started by photosensitization, by direct photochemical reactions, and by autoxidation reactions initiated in various ways. Enzymatic oxidations (e.g., the lipoxygenase reaction) may also be important, as may "indirect oxidations" in which the product of a previous step (such as H_2O_2) oxidizes some substrate. This chapter is an attempt to delineate some of the principal pathways and reactive intermediates and the techniques available to establish their presence.

REACTIVE SPECIES

There are several reactive intermediates which take part in oxidative processes and which must be considered. These include superoxide ion (O_2^- or its conjugate acid, $\text{HO}_2\cdot$), the hydroxyl radical ($\text{OH}\cdot$), alkoxyl radicals ($\text{RO}\cdot$), peroxy radicals ($\text{ROO}\cdot$), and singlet molecular oxygen ($^1\text{O}_2$).

Chain Autoxidation

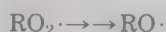
In all of the reactions under discussion, it is important to realize that the primary reaction step may lead to a peroxide, which can in turn initiate radical chain autoxidation (Scheme 1) (Pryor 1982). The chain may result in consumption of a very large number of molecules for each initiating event, and it may be very difficult to establish the initiation step, since it is effectively "buried" by the subsequent free radical steps. The initiator $\text{X}\cdot$ may be an adventitious free radical, or may be formed from a peroxide (see below).



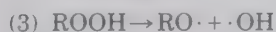
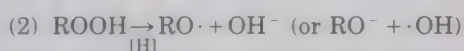
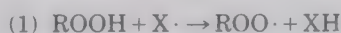
SCHEME 1

Peroxy and Alkoxy Radicals

The ground state of molecular oxygen is a triplet, and thus has some properties of a diradical. In particular, it reacts rapidly with radicals to give peroxy radicals. These are converted readily into the more reactive alkoxy radicals, and both are important chain-carrying species in autoxidation.



There are three principal mechanisms by which a hydroperoxide can start a chain reaction. (1) The hydroperoxide can itself react with a radical ($\text{X}\cdot$) from some source to give a peroxy radical which can take part in a chain. (2) The hydroperoxide can be reduced by a metal ion or another reductant to give an alkoxy radical (or less likely, hydroxyl radical). This is an important role for transition metal ions in these systems. (3) Less importantly at room temperature than at elevated temperatures, the hydroperoxide can break the O—O bond directly to give alkoxy and hydroxyl radicals.



Superoxide Ion

Superoxide ion has been suggested to be important in many cases of biological oxygen toxicity; it is formed by electron transfer whenever oxygen encounters a strong reductant (Fridovich 1978). This reductant may be a metal, a slightly uncoupled electron transport system, or an enzymatic species or some other strong reducing agent.

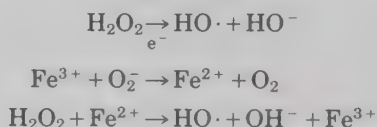


The reactions of superoxide are fairly limited; it does not seem to abstract hydrogen atoms directly except from very strong hydrogen atom donors such as ascorbate (Greenstock and Ruddock 1976); its major mode of action is as a reductant. However, it is in equilibrium with its conjugate acid, the perhydroxyl radical ($\text{p}K_{\text{a}}$ 4.8); the perhydroxyl radical is a considerably more powerful oxidant than superoxide itself (Bielski *et al.* 1983). Since at neutral pH approximately 1% of the O_2^- is present in the protonated form, this species cannot be neglected.



The Hydroxyl Radical

The hydroxyl radical probably is generated from H_2O_2 by a reductant. It was originally believed that superoxide could serve as a reductant in this reaction; however, it is now known that the reaction $\text{O}_2^- + \text{H}_2\text{O}_2$ is too slow to account for significant production of hydroxyl radical (Weinstein and Bielski 1979). However, an indirect reaction giving the same products but involving the intermediacy of metal ions such as iron or manganese is more likely (Scheme 2). Certain chelators can either promote or inhibit the activity of metals.



SCHEME 2

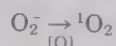
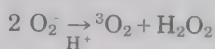
The reactivity of the hydroxyl radical is extremely high; it reacts with all organic substrates within a few collisions (Farhataziz and Ross 1977). Scheme 2 thus provides a route for the production of the extremely reactive hydroxyl radical from the much less reactive superoxide ion. It is not clear why superoxide ion should be required for the generation of a hydroxyl radical, since, in principle, any reductant strong enough to reduce Fe^{3+} could substitute for it in this process (Fee 1982).

Singlet Oxygen

In addition to the ground state (triplet) of molecular oxygen whose role in radical autoxidation reactions has been described above, there is an important excited state of oxygen, the singlet, which has a moderately long lifetime (Foote 1982). It can be produced in photochemical reactions and in some dark reactions as well. Singlet oxygen can react with many organic substrates to give hydroperoxides or endoperoxides. It is more selective than the hydroxyl and alkoxyl radicals, but less so than superoxide ion.

It has also been suggested that the proton-catalyzed dismutation of superoxide ion (which gives H_2O_2 and O_2) could produce some of the oxygen in the singlet state (Khan 1970). Most authors (although not all) now agree that the acid-catalyzed dismutation of superoxide ion gives essentially no singlet oxygen (Aubry *et al.* 1981; Foote *et al.*

1980). However, there is some evidence that certain oxidants can produce singlet oxygen from superoxide ion (Nanni *et al.* 1981).



The dismutation of superoxide ion shown above can also be efficiently catalyzed by the enzyme superoxide dismutase (SOD). This reaction is also known not to be a source of singlet oxygen (Goda *et al.* 1974).

Lifetimes

The lifetimes of the reactive species in "biological" media (which we will not define further at this point except to assume they are aqueous media with concentrations of organic substrate typical of cytosol or else lipid-like regions with high concentrations of hydrocarbon material, typical of membranes) are quite varied. The hydroxyl radical has the shortest lifetime; because of its extremely high rate of reaction with most organic substrates, its lifetime probably does not exceed 10^{-7} to 10^{-10} sec in typical biological media (Farhataziz and Ross 1977). For this reason, its range of diffusion is extremely limited. Alkoxyl radicals are slightly less reactive and have longer lifetimes, and peroxy radicals have lifetimes several orders of magnitude longer still (Howard and Scaiano, 1984). Singlet oxygen has a lifetime which varies from milliseconds in aprotic media to microseconds in water and is capable of diffusing over distances comparable to the thickness of cell membranes (Wilkinson and Brummer 1981). Superoxide ion has complex kinetic behavior since its major loss reaction, as mentioned previously, is dismutation in the presence of a proton source to give H_2O_2 and oxygen. Because this reaction requires two superoxide molecules, it has second-order kinetics. The known rate constants require that the time for half the superoxide to disappear by dismutation varies from milliseconds at millimolar to hours at nanomolar concentrations, although it is very dependent on hydrogen ion (Bielski and Allen 1977). Thus, low concentrations of superoxide can diffuse long distances in the absence of SOD.

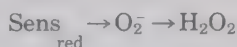
PHOTOSENSITIZED OXIDATIONS

Photochemical routes are very important for formation of the initial peroxide which can serve as initiator (Foote 1976). Direct absorp-

for the type 1 reaction, although they can also sensitize type 2 processes. Porphyrins such as chlorophyll (but not those containing transition metals) are excellent sensitizers for the type 2 process and give a good yield of singlet oxygen.

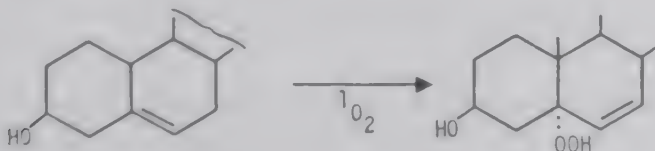
Indirect Reactions

Photosensitized reactions can also lead to indirect oxidation reactions by way of various oxygen radicals. For example, the first step of the type 1 reaction often leads to reduced photosensitizer, which can be reoxidized by oxygen to give superoxide ion or its conjugate acid, the perhydroxyl radical. These species undergo dismutation to produce hydrogen peroxide, and (as mentioned above) radicals produced in the type 1 reaction can also react with oxygen to give peroxy, then alkoxy radicals. Subsequent reactions of these species give further chemistry.



Targets

The biological targets of sensitized photooxygenation are well known from the extensive studies which have been carried out on "photodynamic action," the toxic action of sensitizers, light, and oxygen on living organisms (Foote 1976). Among the principal ones are proteins, with the amino acids histidine, methionine, tryptophan, tyrosine, and cysteine being primary targets. Nucleic acids are also damaged, with guanidine being the principal target. Particularly important for the present subject is damage to lipids, which involves the oxidation of unsaturated compounds such as fatty acids and cholesterol. Reaction of the lipids with singlet oxygen provides hydroperoxides. For example, cholesterol yields a characteristic 5- α -hydroperoxide (see below) which is thought to be diagnostic for singlet oxygen. (Lipid peroxidation is particularly important in rancidity, since the peroxides and radicals derived from them lead to rancidity odors and flavors.) The other target molecules are modified in complex ways. Most of the same substrates are targets in the oxidative deterioration of food, not only by singlet oxygen, but also by some of the other reactive species.

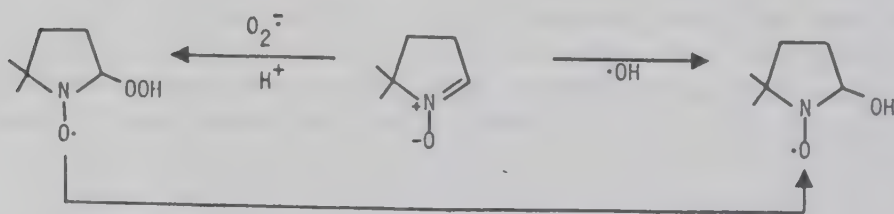


METHODS OF CHARACTERIZING REACTIVE INTERMEDIATES

It is important to develop methods for the specific detection and quantitation of the reactive species which are intermediates in the processes described above. An essential part of this process is to establish not only that the reactive intermediate is present, but that it is actually causing the observed oxidation process, and what fraction of this oxidation is due to the intermediate. As already mentioned, this may be difficult because any one of the reactive species may produce peroxides which will induce radical chain oxidation and obscure the reactions of the initial species. Nevertheless, some progress has been made on this task (Foote 1979; Singh 1982).

Hydroxyl Radical

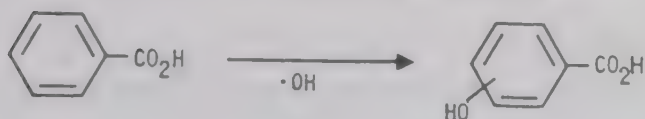
Several different techniques have been used for detection of the hydroxyl radical (Singh 1982). Because of its extreme reactivity, it is very difficult to quantitate its importance. The types of systems which have been used are basically chemical traps and inhibitors. These traps are of several types: There are a number of "spin traps" which react with hydroxyl radicals to give a more stable radical that can be detected by EPR (Janzen 1980). Some of these traps also react with superoxide to give a different adduct; in some cases, this adduct is unstable and rapidly decays to the same adduct produced from hydroxyl radical (Rosen and Rauckman 1984).



Methional is converted to ethylene by $OH\cdot$ (Yang 1969). However, it is likely that ethylene formation is not very specific for hydroxyl radicals, since other radical species have been shown to produce it as well (Pryor and Tang, 1978)



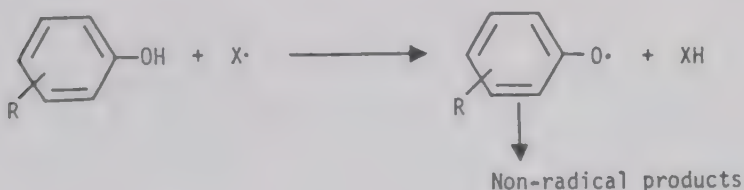
Aromatics such as benzoic acid are hydroxylated and the hydroxylated product can be detected. This appears to be a reasonably specific test for hydroxyl radical (Richmond *et al.* 1981).



Another diagnostic test for hydroxyl radical has been the use of compounds such as alcohols and aromatics which are somewhat more reactive than other substrates and which will therefore inhibit their reactions. The problem is that the hydroxyl radical is so reactive and nonselective that the inhibitors must be used at very high concentration to have any effect. In addition, the question always exists of whether the hydroxyl tests really discriminate between $\text{RO}\cdot$ and $\text{OH}\cdot$. Of the previously mentioned tests, only the hydroxylation of aromatics is probably specific for $\text{OH}\cdot$.

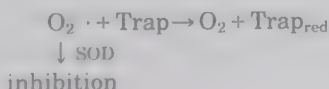
Peroxyl and Alkoxy Radicals

Peroxyl and alkoxy radicals can also be studied by spin trapping. The chain processes in which they are involved can also be inhibited by compounds such as phenolic antioxidants. These compounds break chain processes by reacting with radical intermediates to form non-radical products. Use of these antioxidants to suppress radical chain reactions is often essential if other reactive species are to be studied.



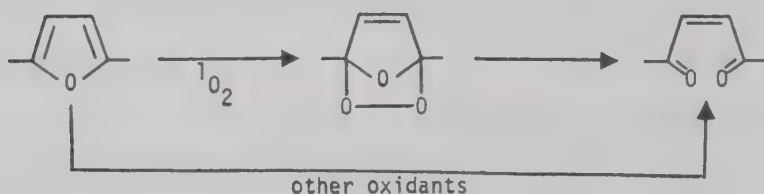
Superoxide Ion

Superoxide ion is usually detected by its reducing properties. An absorbing species such as ferricytochrome *c* or Nitro Blue Tetrazolium is reduced by superoxide; the inhibition of this reduction by superoxide dismutase is used to make the reaction specific for O_2^- . Spin traps can also be used, but the adducts with superoxide ion are not very stable, as mentioned above.

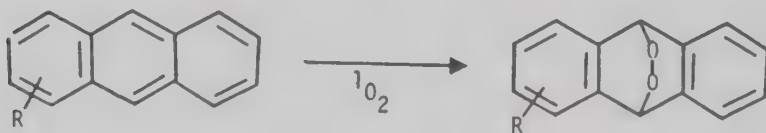


Singlet Oxygen

There are a variety of different methods of detecting singlet oxygen (Foote 1979). Chemical traps are often used, but the product must be specific for singlet oxygen if this method is to be useful. Furans were one of the first classes of compounds to be used as traps; they are oxidized to the diketone as the final product, which is the one usually isolated. The reaction proceeds via an intermediate endoperoxide, which cannot usually be isolated. Formation of the diketone is not a very specific test for the presence of singlet oxygen, since a wide variety of other oxidants convert furans to the same product.

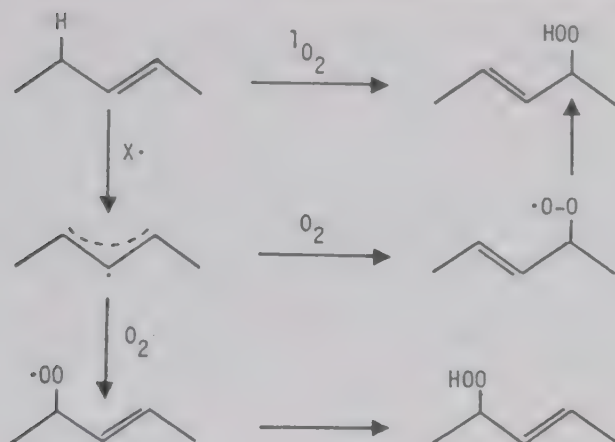


Dienes, olefins, and aromatics provide more distinctive tests. For example, a number of anthracene derivatives with various solubilizing substituents produce endoperoxides with singlet oxygen. These products appear to be specific.

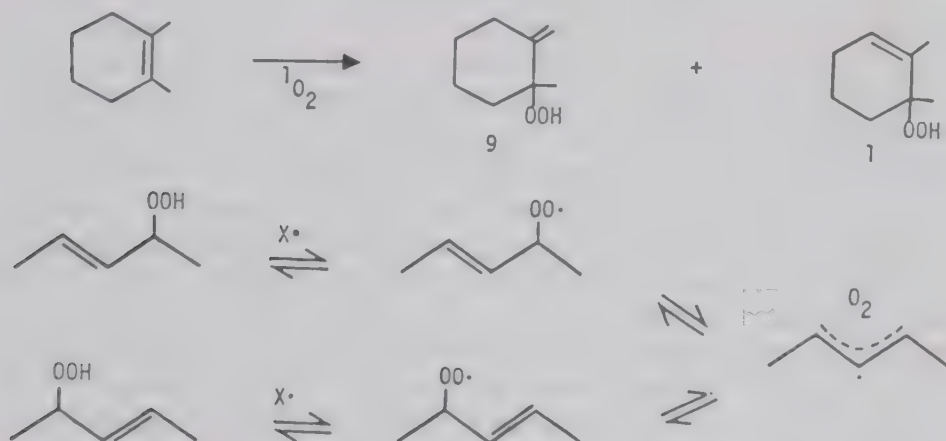


Olefins have also been used; they undergo the so-called "ene reaction" in which an olefin with an allylic hydrogen is oxidized to a hydroperoxide. This hydroperoxide is formed very specifically with a shift of the double bond to the allylic position. In contrast, the products of autoxidation can form product by attack at either or both of the resonance-stabilized radical sites, as shown below.

For example, dimethylcyclohexene gives a product ratio with singlet oxygen which is very different from that of free radical autoxidation. However, it is important to realize that the hydroperoxides may undergo rearrangement, particularly in the presence of free rad-

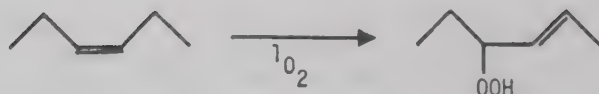


icals. Thus, the initially formed specific hydroperoxide may rearrange by the mechanism shown below to the free radical product mixture (Porter, Chapter 5, this volume).



A particularly distinctive substrate for singlet oxygen is cholesterol; the 5- α hydroperoxide which is formed (see above) appears to be specific (Ansari and Smith 1979). This product is subject to the rearrangement discussed above; nevertheless, if the 5- α product is detected, it is a distinctive test for singlet oxygen. Oxidation of fatty acids has also been used; the products are distinctly different from those of free radical autoxidation (Thomas and Pryor 1980).

In addition to chemical traps for singlet oxygen, various inhibitors have been used. β -Carotene is a very powerful inhibitor of singlet oxygen, although soluble only in lipid media. It has been shown to



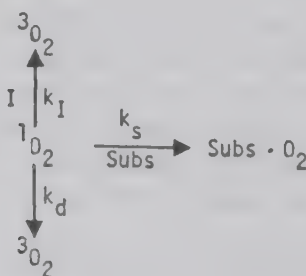
quench singlet oxygen at a diffusion-controlled rate, so that even very low concentrations are sufficient to inhibit reactions of singlet oxygen. It has been recognized for some time and recently demonstrated conclusively that β -carotene is also a radical scavenger (Burton and Ingold 1984); thus, carotene inhibition may not be specific for singlet oxygen. Many other compounds also quench singlet oxygen, and many of them have been used to test for its presence. It should be recognized that all singlet oxygen quenchers are compounds of low oxidation potential and would be expected to react with other strong oxidants.



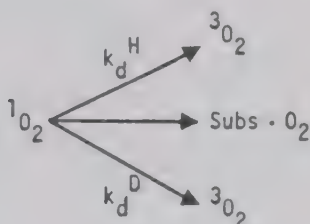
Various phenols both react with and quench singlet oxygen; α -tocopherol is a particularly reactive substrate and quenches singlet oxygen with a rate near $10^9 M^{-1} \text{sec}^{-1}$; it reacts to give stable products at a somewhat lower rate constant (Wilkinson and Brummer 1981). Tocopherol is another compound which is a powerful radical chain terminator, thus not likely to be very suitable for characterizing singlet oxygen reactions because of its profound effect on autoxidation.

In the ideal case, in homogeneous solution, it is possible to set up a kinetic scheme using inhibitors or traps to characterize singlet oxygen definitively; rate constants for reaction of singlet oxygen with many substrates and inhibitors are known, as are its rate constants for decay in many solvents (Wilkinson and Brummer 1981). Thus, the percentage inhibition of reaction of the target molecule which would be caused by an inhibitor can be predicted and compared with that observed.

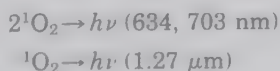
Another method of characterizing singlet oxygen as a reaction in-



intermediate is to study the effect of replacing H_2O by D_2O . This method rests on the observed fact that the lifetime of singlet oxygen is substantially (10–20 times) longer in D_2O than H_2O ; thus, singlet oxygen reactions would be expected to be more efficient in D_2O than in H_2O . It is important to recognize that this method has limitations: (1) for the isotope effect to be observed, the lifetime of singlet oxygen must be limited by solvent. If all the singlet oxygen is being scavenged by substrate, no effect will be observed. (2) The superoxide ion lifetime is also longer in D_2O than H_2O , and thus superoxide reactions should also go more rapidly in D_2O than H_2O ; this fact has frequently been overlooked (Foote *et al.* 1984).



Luminescence has also been used extensively for characterization of singlet oxygen. There are two luminescence processes from singlet oxygen: One is the so-called "dimol luminescence" in which two moles of singlet oxygen give a single quantum of light of twice the energy of either molecule. This emission occurs at 634 and 703 nm. There is also a direct emission at 1.27 μm . Both these luminescence mechanisms are very inefficient and the infrared emission is very difficult to detect. There are many species that are capable of low-level chemiluminescence; if the specific wavelengths of singlet oxygen emission are not observed, visible chemiluminescence is meaningless (Denecke and Krinsky 1977). The 1.27 μm emission (at least so far) appears to be more specific. Although this luminescence is extremely weak, it can be detected in some types of photolysis experiments. Its precise lifetime and emission wavelength can be measured under these conditions (Ogilby and Foote 1983; Hurst and Schuster 1983). Photochemical experiments produce very large amounts of singlet oxygen. Whether the 1.27 μm emission will ever be useful for detecting singlet oxygen in systems which are undergoing nonphotochemical oxidation is questionable at the present.



SUMMARY

This article has sketched a few of the techniques which can be used for characterizing reactive species that may be involved in oxidation reactions. There are no simple answers to the questions posed in many cases; all the methods described have limitations and probably a combination of them should be used, and always with the recognition that the initial process is likely to be amplified by radical chain autooxidation.

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Metal-Catalyzed Reactions of Organic Compounds

*Arthur E. Martell*¹

INTRODUCTION

It is the purpose of this chapter to describe the various ways by which metal ions and metal complexes may catalyze reactions of organic compounds. Descriptions of reaction pathways and the mechanisms involved will be illustrated by examples of organic compounds present in foods or closely related compounds.

In principle, the reactions involved in metal ion catalysis may be subdivided into two broad areas: (1) reactions in which the metal ion coordinates functional groups of the organic compound having Lewis base properties, resulting in polarization of these functional groups and in the activation of adjacent sites in the molecule; and (2) electron transfer reactions in which the metal ion goes from a higher to a lower oxidation state or from a lower to a higher oxidation state, resulting in corresponding oxidation or reduction reactions in the organic compound to which the metal is coordinated. The initial changes in the organic compound produced by the metal ion may be followed by additional reaction steps, depending upon the structure of the compounds. These reactions include rearrangements, elimination of elec-

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tronegative groups, reaction with other molecules present in the system, and even carbon-carbon bond fission reactions.

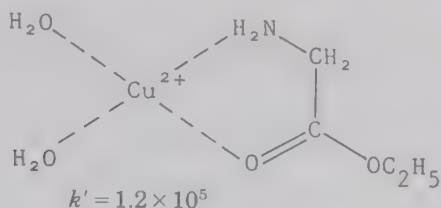
Examples are known of reactions in which a metal ion coordinates simultaneously to both an oxidant and a reductant, thus providing a pathway for electron flow and leaving the metal ion essentially unchanged. This type of reaction is facilitated by unsaturation and conjugation in the organic ligands. Alternatively, a metal ion may act as a carrier by taking one or more electrons from a reductant and transferring it to the oxidant by dissociation from the former and coordination with the latter. Atom transfer processes may be promoted by metal ions through the formation of intermediate complexes, with the atom being inserted into the organic molecule.

The reaction types just cited are metal ion-promoted stoichiometric processes. The metal ions or metal complexes involved in these reactions become catalytic when the reaction conditions are favorable for regeneration of the original metal ion or complex after completion of the stoichiometric reaction. Thus, ionic reactions promoted through metal coordination and polarization of an organic molecule may terminate by dissociation of the metal ion, which then is free to begin the process again. In redox systems the oxidation or reduction of an organic substrate by a metal ion would normally terminate when all of the metal ion available has reacted. However, an appropriate reductant or oxidant in the reaction medium may then convert the metal ion to its original state, thus forming the basis for a catalytic process.

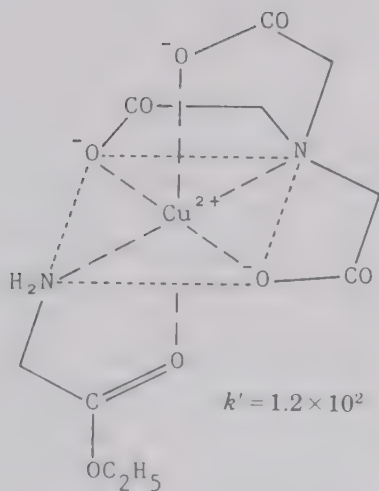
IONIC REACTIONS PROMOTED BY METAL IONS

Metal Ion Activation of Organic Compounds Toward Nucleophilic Attack

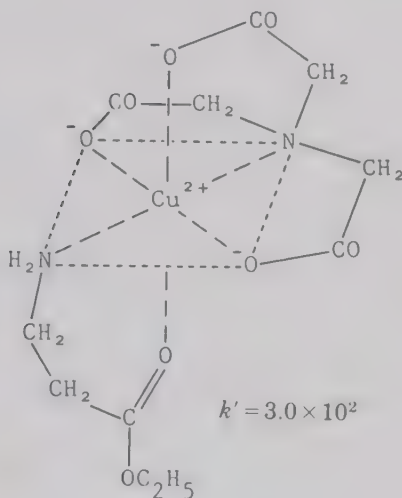
Metal-Catalyzed Ester Hydrolysis. The carbonyl oxygen atoms of esters are generally weak donors and form relatively unstable complexes with metal ions both in aqueous and nonaqueous solutions. However, metal ion activation of the ester carbonyl carbon toward nucleophilic attack is considerable, due in part to the fact that the product of the reaction is a much more stable coordination compound, thus leading to the formation of a relatively stable transition state. The rate constants determined (Eq. 1; Hopgood and Angelici 1968A, B) for the hydrolysis of amino acid esters in the presence of copper (II) ions and complexes provide insight into the catalytic effects involved in these reactions. The aquo copper(II) ion interacts strongly with the ethylglycinate (formula 1) and is an effective catalyst for nucleophilic attack of the carbonyl carbon by hydroxide ion and the resulting hydrolysis of the ester. Coordination of copper(II) with a sec-



1



2



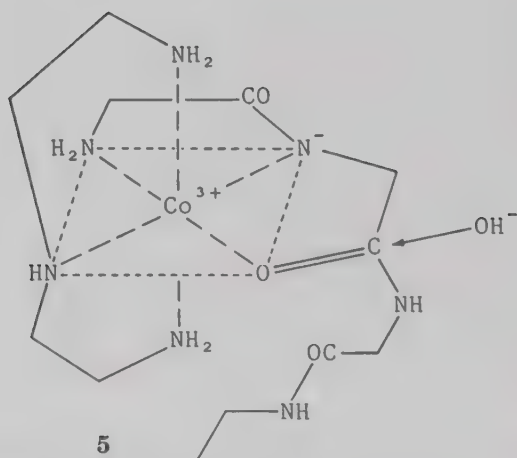
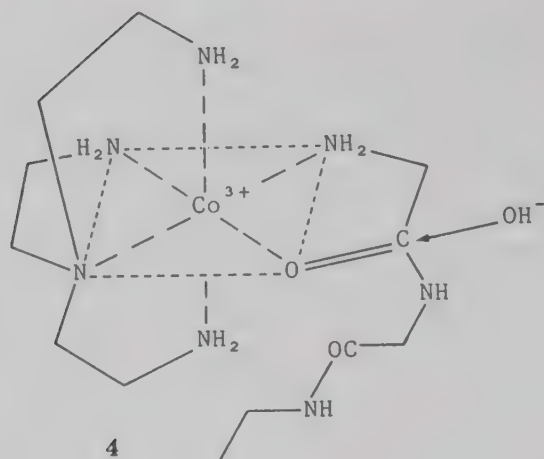
3

$$k_{\text{obs}} = k_0 + k_1[\text{OH}^-] \quad (1)$$

$$k^1 = k_1 (\text{complex}) / k_1 (\text{metal-free})$$

ondary ligand, however (formula 2), such as NTA, satisfies a considerable fraction of the coordination tendencies of the metal ion, so much less remains to promote ester hydrolysis, resulting in a lowered reaction rate constant. If the coordination interaction of the metal ion and the amino acid substrate is further reduced, as in the case of the ethyl ester of β -alanine (formula 3), the hydrolysis constant is reduced even further. In the latter case, one might argue that the copper ion should polarize the carbonyl group as strongly as it does with 2. While this may be true, it is apparent that the probability of coordinating the carbonyl oxygen in 3 would be somewhat lower than in 2 because of the formation of a less stable chelate ring.

Peptide and Amide Hydrolysis. The selective cleavage of an amino acid from the N-terminal position of a polypeptide is catalyzed by cobalt(III) complexes of tetradentate polyamines such as that of tria-

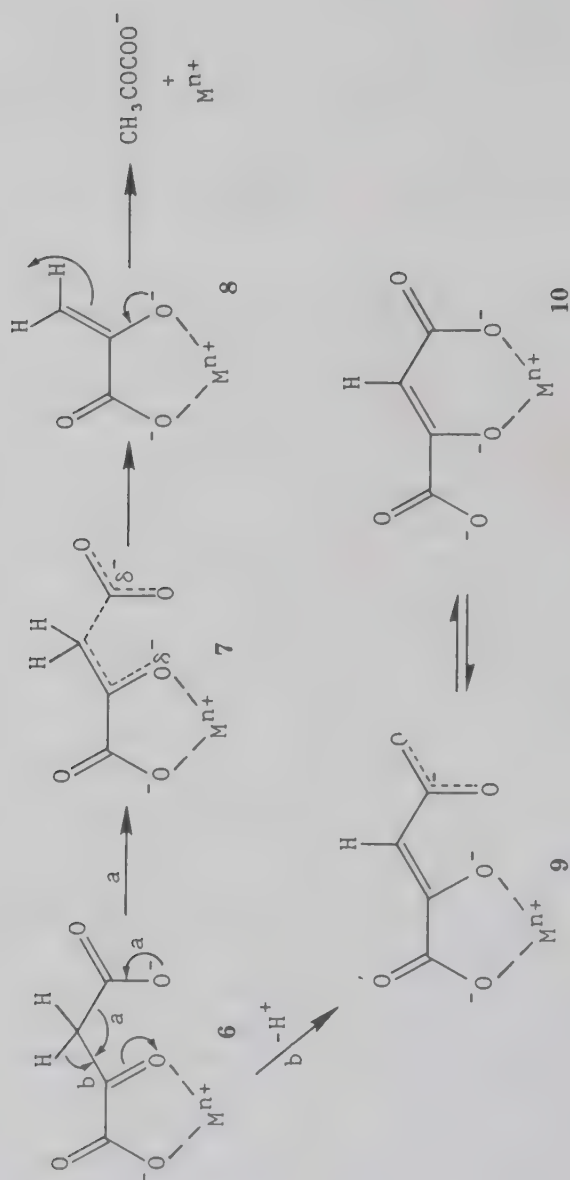


minotriethylamine, illustrated by **4** (Collman and Buckingham 1963; Kimura *et al.* 1970; Buckingham and Marzilli 1967). Combination of the cobalt(III) catalyst with only the terminal amino group and the adjacent carbonyl oxygen is due to the fact that these two positions are the most strongly coordinating adjacent sites available in the polypeptide and because there are only two uncoordinated (aquo) positions remaining in the cobalt(III)–polyamine complex. Activation of the terminal peptide linkage indicated in **4** toward nucleophilic attack by hydroxide ion results in the observed selective catalysis. The cobalt(III) complex of a triamine with a polypeptide chain of the type indicated by **5** would give less selective and weaker catalysis because of the involvement of other parts of the polypeptide chain in coordination.

The type of catalysis indicated by **4** is analogous to the catalytic action of metal ion-activated proteolytic enzymes such as carboxypep-

tidase and aminopeptidase. In these systems the metal ion is coordinated to the protein of the enzyme by functional groups derived from the side chains of the substituent amino acids, but has additional free coordination sites for combination with two or more functional groups of a peptide undergoing selective cleavage. Stereospecificity in these enzyme systems is achieved by functional groups of the protein at the active site which serves to position the substrate so as to direct metal catalysis at the desired carbonyl oxygen. The additional functional groups at the active site also serve to accelerate the reaction by coordinating the products resulting from hydrolytic cleavage.

Decarboxylation. The decarboxylation of α -keto acids that also have β -keto acid functions 1947; (Speck 1948; Gelles and Hay 1958; Gelles and Salama 1958A; Gelles and Salma 1958B; Hay 1965) is a good example of a reaction type which is strongly catalyzed by metal ions as indicated by formulas 6–8 (Scheme 1). The complex of oxaloacetic acid indicated by 6 is the weakest of the complexes that may be formed from the available functional groups in this ligand. Formulas 9 and 10 indicate more stable configurations and result from the enolization of a proton from the carbon atom between the carbonyl and terminal carboxylate functional groups. Although it is a minor species in solution, the weak complex (6) is the only catalytic intermediate for decarboxylation. Polarization of the carbonyl oxygen by the metal ion leads to accumulation on the coordinated oxygen of the negative charge derived from bond breaking between the terminal carboxylate and the adjacent methylene group. This electron pair shift gives rise to the transition state (7) and the unsaturated complex (8). The latter is reprotonated to form pyruvic acid and liberates the aquo metal ion, which is then free to repeat the cycle. Since the equilibrium of the final protonation step is strongly in favor of pyruvic acid, this final step is essentially irreversible, resulting in gradual conversion of the whole system to the decarboxylation product. Thus, equilibria involving the side reactions to form 9 and 10 are gradually shifted back through the less stable complex (6) and the metal-catalyzed decarboxylation goes to completion. The relative catalytic effects of a number of metal ions ($\text{Ca}^{2+} < \text{Mn}^{2+} < \text{Co}^{2+} < \text{Ni}^{2+} < \text{Zn}^{2+} < \text{Cu}^{2+}$) have been interpreted as providing supporting evidence for the decarboxylation mechanism indicated by $6 \rightarrow 7 \rightarrow 8$ (Hay 1958). This sequence of metal ion activity does not correlate well with the relative stabilities of the corresponding metal chelates of oxaloacetic acid, but does show a linear correlation with the stabilities of the corresponding metal oxalates. This is considered evidence for the formation of the transition state (7), which represents the rate-determining step in the de-



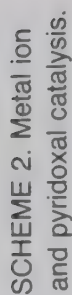
SCHEME 1

carboxylation reaction. Since the structure of **7** resembles that of the corresponding oxalate complexes (seen more clearly in formula **8**), the greater the tendency to form **7** the more rapid one would expect the resulting decarboxylation reaction to be.

It should be noted that metal chelate structures such as **10** stabilize the carboxylate group in the ligand rather than promote decarboxylation. The coordinate bond between the metal ion and the carboxylate oxygens tends to polarize electron pairs in the direction opposite to what would be needed for decarboxylation. Thus, coordination of the carboxylate group in any way, including protonation to give the acid form, would be expected to inhibit decarboxylation.

Metal Ion-Catalyzed Reactions of Pyridoxal with Amino Acids. Metal ions help to promote the pyridoxal-catalyzed reactions of amino acids in two ways: (1) by increasing the stability of the intermediate Schiff base and increasing its concentration in solution, and (2) by promoting the electron shifts necessary to carry out pyridoxal-catalyzed Schiff base reactions such as transamination, dealdolization, and β -elimination of electronegative groups. All of these reactions seem to involve the formation of a carbanion type of intermediate containing a delocalized negative charge adjacent to the azomethine nitrogen (Martell 1982). This intermediate is stabilized by coordination of the azomethine nitrogen with the metal ion, as indicated by **14**. The charge on this carbanionic intermediate may be relieved by loss of the electronegative group with its bonding electron pair from the β position of the amino acid (**15**) or by reprotonation in the position α to the aromatic ring, resulting in the formation of the Schiff base of pyridoxamine and the corresponding α -keto acid (**17**). This reaction sequence results in transamination of the initial amino acid (**11**) to the corresponding keto acid. Reprotonation of the carbanionic intermediate (**14**) at the α position of the amino acid residue results in the formation of the initial amino acid which has been racemized (if the amino acid was chiral) or, in any case, which has undergone proton exchange at the α position. The carbanionic type of intermediate may also be formed by the breaking of the carbon-carbon bond rather than dissociation of a proton to give intermediate **16**, which leads to the formation of **14**. Carbon-carbon bond fission is possible only if an electron pair is available from the substituent in the β position. Such a substituent is most frequently a hydroxyl group which is converted to the corresponding carbonyl compound on loss of a proton.

Although most enzymes for which vitamin B₆ (pyridoxal) is a cofac-



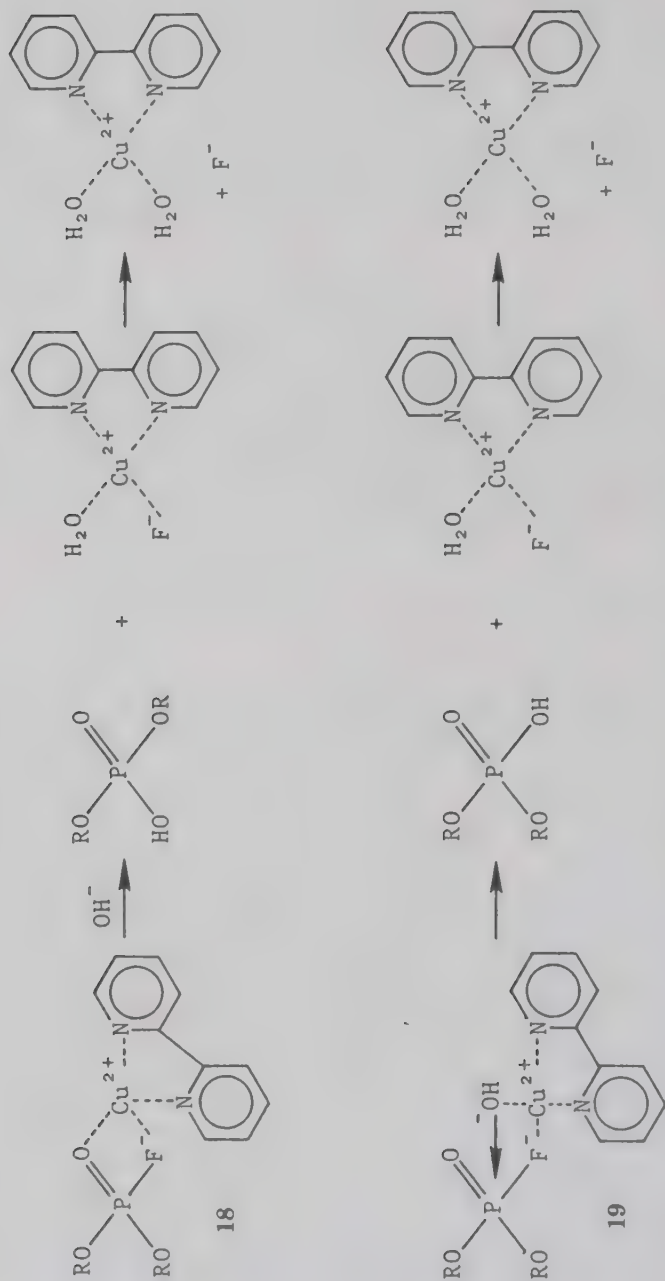
tor do not require metal ions for activation, the reaction sequence in the natural systems are similar to those shown by formulas 12–17 (Scheme 2), with a proton coordinated to the azomethine nitrogen to promote the electron pair shifts that are necessary to carry out these reactions. For some of these proton-catalyzed reactions, such as transamination, it is thought (Martell 1982) that protonation at a second site, the pyridine nitrogen, further accelerates the reaction by pulling electrons away from the amino acid residue and assuring re-protonation at the aldehydic carbon to form a pyridoxamine residue.

Hydrolysis of Fluorophosphosphonates and Fluorophosphates. The hydrolysis of nerve gases such as SARIN (isopropylmethylphosphonofluoridate) and DFP (diisopropylphosphofluoridate) is greatly accelerated by certain metal complexes such as those of copper(II) with bidentate polyamines (Courtney *et al.* 1957; Martell *et al.* 1957; Martell 1963; Gustafson and Martell 1962; Gustafson *et al.*, 1963). The rate law (Eq. 2),

$$k_{\text{obs}} = k_{\text{CuL}}[\text{CuL}][\text{OH}^-] + k_{\text{OH}}[\text{OH}^-] + k_{\text{H}_2\text{O}}, \quad (2)$$

shows a third-order rate constant for metal catalysis, indicating that the rate is dependent on the concentration of the substrate, the concentration of the metal complex, and the hydroxide ion concentration. The additional rate constants shown in the rate expression are smaller and become important only at very high pH or in the absence of metal catalysts. The reaction mechanism consistent with this rate law is indicated below (Scheme 3, formula 18), which involves activation of the phosphorus double bond through coordination with the metal ion (18), thus rendering the phosphorus center more susceptible to attack by hydroxide ion which displaces the fluoride through an $\text{S}_{\text{N}}2$ type of reaction. The fluoride ion may be displaced directly to the solution or may reside temporarily coordinated to the copper ion, as indicated. This mechanism is indistinguishable from one involving second-order attack of the fluorophosphonate by the hydroxo-metal complex, indicated by formula 19. According to this concept, the metal catalyst acts through a "push-pull" type of reaction in which the hydroxide ion is donated to the phosphorus center, while the fluoride ion is transferred to the metal center. The corresponding rate law (Eq. 3),

$$k_{\text{obs}} = k_{\text{CuLOH}}[\text{CuLOH}^+] + k_{\text{OH}}[\text{OH}^-] + k_{\text{H}_2\text{O}} \quad (3)$$



SCHEME 3. Metal chelate-catalyzed hydrolysis of fluorophosphates.

where

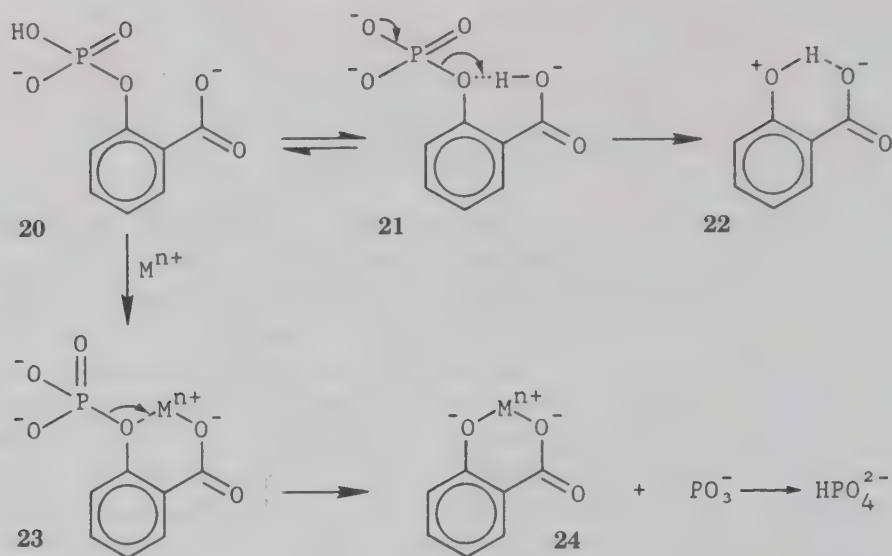
$$k_{\text{CuL}} = k_{\text{CuLOH}} K_{\text{CuLOH}} \quad (4)$$

$$K_{\text{CuLOH}}^{\text{CuL}} = \frac{[\text{CuLOH}^+]}{[\text{CuL}^{2+}][\text{OH}]} \quad (5)$$

is directly related to the third-order expression through the hydrolysis constant of the metal complex catalyst (Eqs. 4 and 5). This situation, whereby second-order attack by a coordinated group is indistinguishable from a corresponding third-order reaction involving metal complex activation of the substrate and simultaneous attack by the free nucleophilic group, is often encountered in metal complex catalysis. The transfer of the hydroxide ion from the coordination sphere of the metal catalyst, indicated by formula 19, is an attractive one. The catalytic effect of the metal ion is achieved by making available the hydroxide ion (a strong nucleophilic reagent) under conditions (i.e., in neutral solution) whereby the hydroxide ion otherwise does not exist in appreciable concentrations.

An interesting example for which this type of mechanism has also been suggested is the nucleophilic attack on carbon dioxide by hydroxide ion at the active site of the enzyme carbonic anhydrase. Here again the alternative choices are activation of carbon dioxide through coordination with the metal center with simultaneous attack by hydroxide ion from solution, or transfer of hydroxide ion from the coordination sphere of Zn(II) to the carbon atom of carbon dioxide by nucleophilic attack.

Hydrolysis of Salicyl Phosphate. Metal ions strongly catalyze the hydrolysis of salicyl phosphate through the formation of a stable metal chelate as the driving force for the reaction. In metal-free systems, hydrolysis of salicylphosphate is strongly catalyzed by protonation of the phosphorus ester oxygen as indicated by formulas 20–22 (Scheme 4). Replacement of the proton in 20 and 21 by a metal ion such as copper(II), indicated by formula 23, results in about a 10^4 increase in the rate of hydrolysis (Murakami and Martell 1964). This increase may be due in part to the fact that the active intermediate (21) is a relatively minor species in proton-catalyzed hydrolysis, whereas the corresponding structure, indicated by 23, for metal ion-catalyzed hydrolysis is probably a major species. In any case, the formation of a stable metal chelate by generating a negative charge on the phenolate oxygen in 24 is a major driving force for the reaction. At high pH where the phosphate ester is completely deprotonated, the lack of proton catalysis results in very slow hydrolysis of the salicyl phosphate ester. Under these conditions, metal ions provide a means of



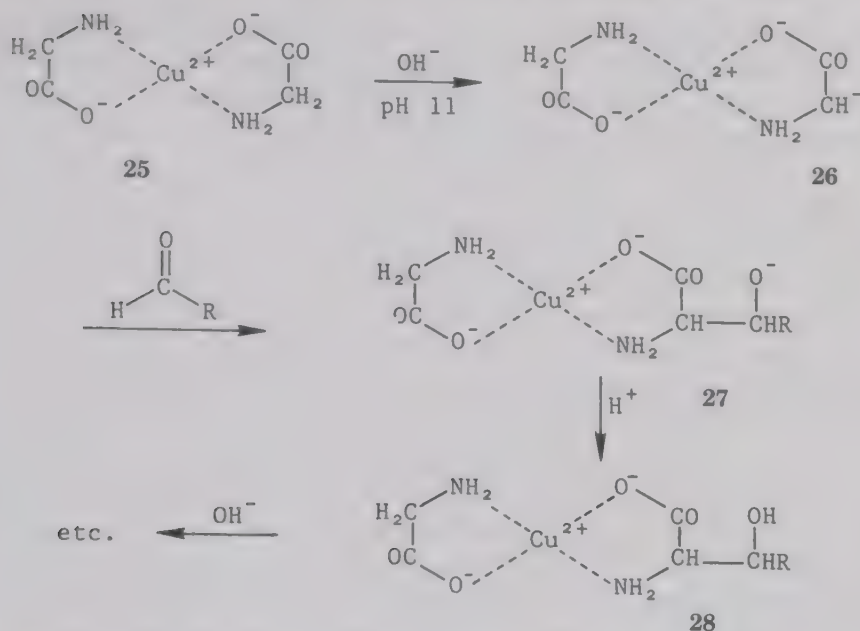
SCHEME 4. Metal ion-catalyzed hydrolysis of salicyl phosphate.

promoting the hydrolysis of the type indicated in **23** with a corresponding acceleration of the rate by a factor of about 10^8 . Because of the fact that metal ions may provide positive centers for coordination of completely dissociated reactants under conditions where protons are not available, metal ions are sometimes referred to as super protons with regard to this type of catalytic effect.

Metal Ion Activation of Organic Molecules toward Electrophilic Attack

Although coordination by a metal ion would generally be considered to increase the positive charges of the positive centers in an organic molecule and thus promote nucleophilic attack, the displacement of one or more protons by the metal ion would increase the negative charge of the coordinated ligand and thus increase its susceptibility to reaction by an electrophilic reagent. An example of this kind of catalysis is the condensation of α -amino acids with carbonyl compounds. Generally, in the absence of metal ions, α -amino acids do not undergo aldol condensation reactions or do so with great difficulty. The copper(II) chelate of glycine, on the other hand, reacts with formaldehyde or acetaldehyde to produce the corresponding copper chelates of serine and threonine, as indicated by the reaction sequence in Scheme 5 (**25–28**) (Akabori *et al.* 1959; Sato *et al.* 1957).

There are many other examples of electrophilic reactions of organic



SCHEME 5. Metal ion-catalyzed electrophilic substitution of glycine.

compounds promoted by metal ion coordination through proton dissociation. Thus, Pederson (1948A, B) reported the catalysis of bromination of the central carbon atom of β -diketone chelates of several metal ions. The catalytic activities of the metal ions investigated seem to parallel the stabilities of the β -diketone chelates formed. Other electrophilic substitution reactions reported for chelated β -diketones include nitration, formylation, and acylation (Reihlen *et al.* 1925; Djordjevic *et al.* 1959; Klukber 1960; Collman *et al.* 1960, 1961, 1962; Collman 1963).

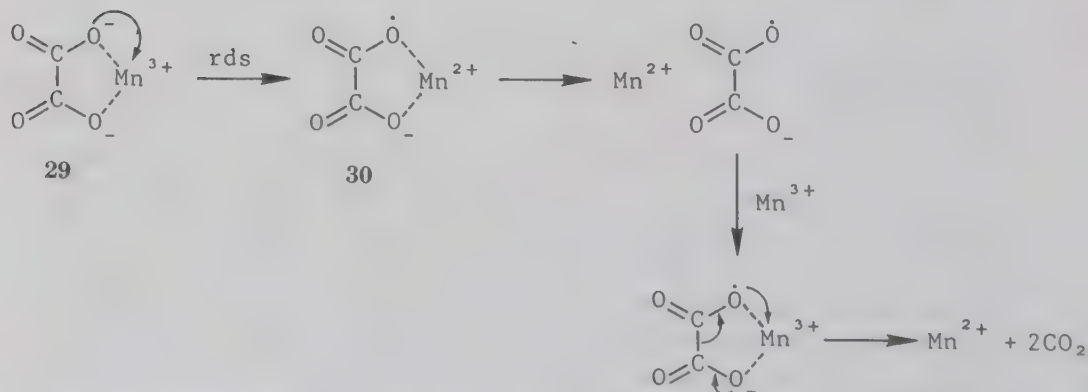
METAL-CATALYZED OXIDATION OF ORGANIC COMPOUNDS

Catalysis of the redox reactions of organic compounds by metal ions and metal complexes is a broad and extensive subject which is far beyond the scope of this chapter. Because of the fact that most metal-catalyzed oxidation reactions of organic compounds of interest to food technology involve metal activation of molecular oxygen, this type of metal catalysis is considered in some detail. In many cases, metal dioxygen complex formation has been demonstrated or inferred in the oxidation and oxygenation of organic substrates, and attention is given to possible mechanisms of such reactions. While molecular oxygen is

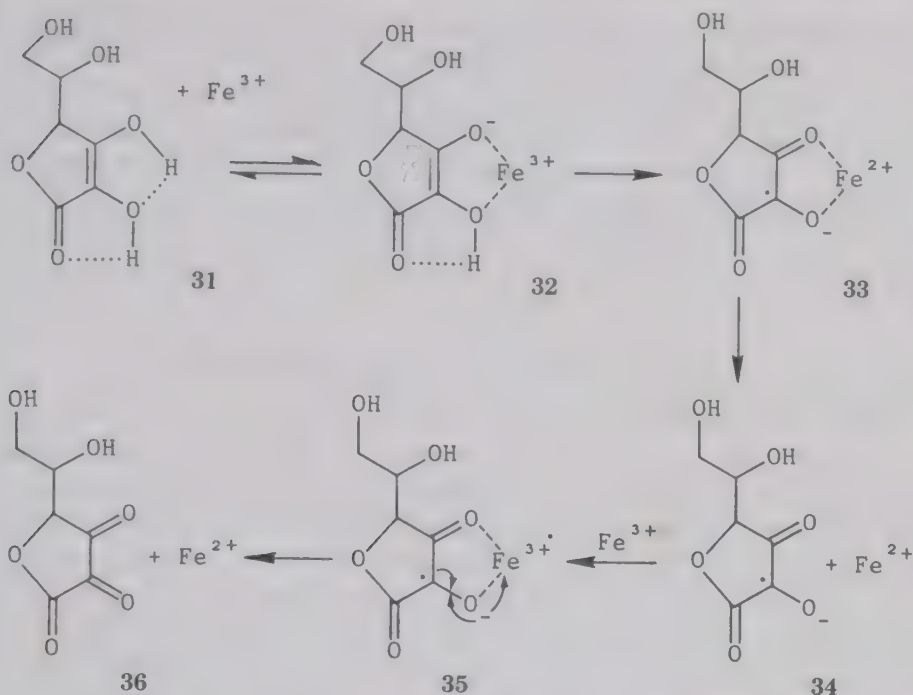
usually the primary oxidant, hydrogen peroxide is frequently produced as an intermediate and becomes available for further oxidation reactions. Some of the metal-catalyzed oxidations of organic substrates by peroxide will therefore be considered.

Stoichiometric Oxidation by Metal Ions

Since most organic molecules involve electron pair bonding, oxidation to stable species generally occurs by the removal of two electrons in each step, although there are some exceptions involving structures that stabilize the intermediate free radicals. Redox reactions of metal ions, on the other hand, usually occur by one-electron transfers, so that two metal ions are usually required for the conversion of an organic compound to the next stable oxidized form. A classic example is the oxidation of oxalate by manganese(III) ion, indicated by **29** and **30** (Scheme 6). The first redox reaction results in the formation of an unstable free radical which then must dissociate and recombine with a second manganese(III) ion for completion of the reaction (Taube 1947, 1948; Duke 1947). It is seen that the reaction between manganese(III) and oxalate terminates when the oxidant or the reductant has been completely reacted. This type of system becomes catalytic, requiring only a trace of manganese ion in the presence of a secondary oxidant such as halogen or dioxygen, which is capable of reoxidizing the catalyst from Mn(II) to Mn(III). Similar studies have been carried out on the oxidation of glycols (Duke and Bremer 1951; Duke and Forist 1949) and glycerol by Ce(IV) (Guilbault and McCurdy 1963) and the oxidation of ethylenediamine (Anbar *et al.* 1963) and glycine (Anbar 1965) by Tl(II) ions.



SCHEME 6. Oxidation of oxalate by Mn(III) ion.

SCHEME 7. Oxidation of ascorbic acid by Fe(III) .

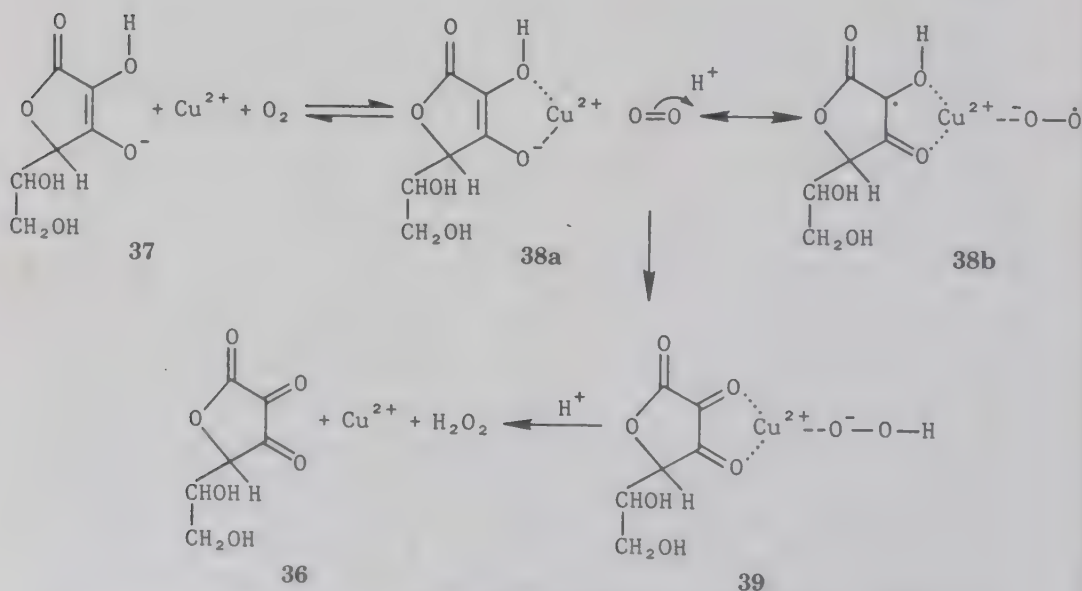
Another example of the direct stoichiometric oxidation of an organic compound by a metal ion is the oxidation of ascorbic acid by ferric ions, illustrated by the reaction sequence involving **31–36** (Scheme 7) (Taqi Khan and Martell 1967A). A similar reaction scheme would describe the oxidation of ascorbic acid by Cu(II) ion. The monoprotonated metal chelate illustrated by **32** has been identified as the reactive intermediate for metal-catalyzed oxidations in acid solution. The reactions in alkaline solutions are extremely rapid and have not been subjected to detailed study. Here again it is seen that two successive one-electron transfers from the reductant to the metal ion are necessary to convert the ascorbic acid to a stable oxidized material, dehydroascorbic acid (**36**). In this case the intermediate free radicals are stabilized by the availability of a number of sites on the molecule for delocalization of the odd electron. The stoichiometric stepwise redox reactions of the type described above may also be carried out with Fe(III) and Cu(II) chelates as the oxidizing agents (Taqi Khan and Martell 1967B). Because of the fact that formation of chelates tends to stabilize the higher oxidation states of coordinated metal ions, oxidation by the coresponding metal chelates is generally very

much slower than the corresponding reactions for which the aquo metal ion is the primary oxidant.

These stoichiometric reactions (31–36) form the basis for catalytic systems when a primary oxidant is present that is capable of reoxidizing the reduced form of the metal to its initial oxidized state. The most common and important examples of this type of catalytic system involve molecular oxygen as the oxidant and are described in some detail.

Metal Ion and Metal Chelate Catalysis of Oxidation by Molecular Oxygen

Ascorbic Acid Oxidation. The oxidation of ascorbic acid to dehydroascorbic acid by molecular oxygen is very strongly catalyzed by Cu(II) and Fe(III) ions. The observed reaction rate is first order in the concentration of the mono anion of ascorbic acid, first order in metal ion concentration, and, over a considerable range of dioxygen concentration, first order in the concentration of dioxygen. These observations have led to the postulation of a metal ascorbate dioxygen complex as the reactive intermediate for electron transfer, and the corresponding proposed reaction scheme is illustrated by 37–39 (Scheme 8) (Taqui Khan and Martell 1967A). It should be noted that the dioxygen complex illustrated by 38a,b is rather unusual in that oxygen

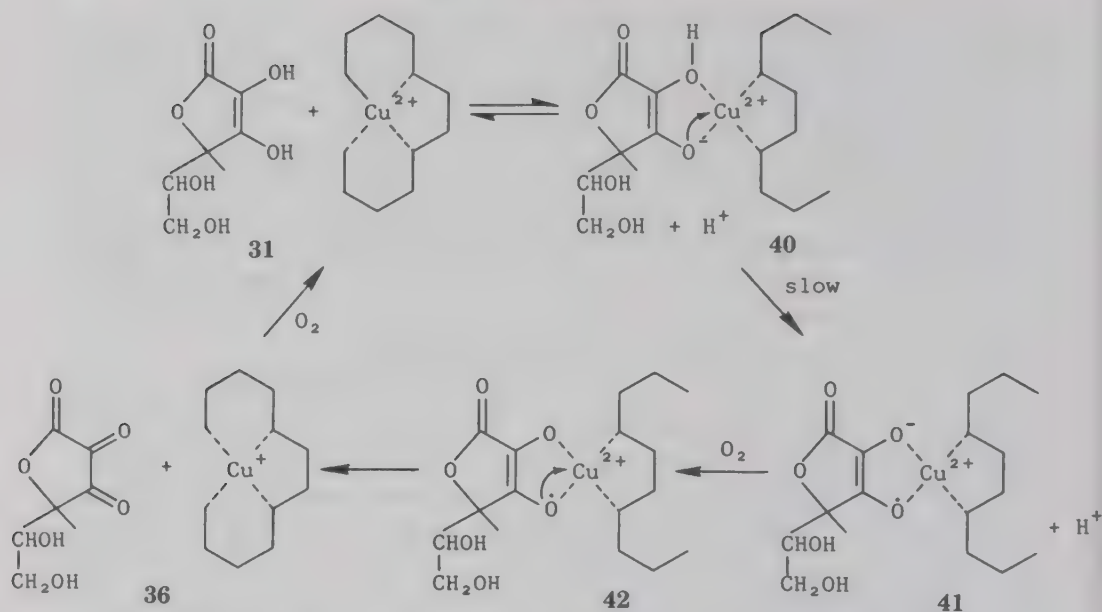


SCHEME 8. Copper(II) ion-catalyzed oxidation of ascorbic acid by dioxygen.

complexes are usually formed from metal ions in their lower valent state. In this case the partial reduction of the Cu(II) is achieved through coordination with the ascorbate anion so that the metal chelate as a whole represents a reduced oxidation state which can promote partial electron transfer to the dioxygen to provide sufficient transient stability to form the reactive intermediate indicated. From this point of view the proposed dioxygen complex represents an intermediate stage in the two-electron oxidation of ascorbic acid to dehydroascorbic acid. The suggestion of a reactive intermediate, with the bonding indicated by **38**, was first made by Hamilton (1969).

The oxidation of ascorbic acid by molecular oxygen may also be catalyzed by many other metal ions capable of undergoing redox reactions between two valance states. In most cases, the reaction sequence is analogous to the stoichiometric reactions described in the section on stoichiometric oxidation by metal ions or to the mechanism involving dioxygen complex formation indicated above for Cu(II) and Fe(III) ions. Dioxygen complex formation has been implicated in the vanadyl- (Taqui Khan and Martell 1968) and uranyl- (Taqui Khan and Martell 1969) catalyzed oxidation of ascorbic acid. On the basis of the experimental results and corresponding mechanisms described above, it is apparent that many other metal ions are capable of promoting the oxidation of ascorbic acid by molecular oxygen. It is also apparent that analogous mechanisms may also apply to ascorbic acid oxidation involving metal catalysis in which the primary oxidant is a reagent other than molecular oxygen, such as halogens, hydrogen peroxide, nitrite ion, and many others.

Metal Chelate-Catalyzed Oxidation of Ascorbic Acid. The oxidation of ascorbic acid by molecular oxygen with various metal chelate compounds of Cu(II) and Fe(III) as catalysts occurs at much slower rates than the corresponding metal ion-catalyzed reactions. The observed rates vary inversely with the stabilities of the metal chelates involved and are first order with respect to the metal chelate compound and with respect to the ascorbate monoanion. In these cases, however, there is no dependence on the oxygen concentration. Therefore, oxygen merely serves as the primary oxidant which functions by reoxidation of the lower valence form of the metal chelate to the reactive higher valence form. Electron transfer probably occurs through one-electron steps from the ascorbate anion to the coordinated metal ion, as indicated in the reaction sequence illustrated in Scheme 9 (**40–42**). The question of whether the reaction occurs through an inner sphere or an outer sphere mechanism has not been investigated, and the reaction mechanism shown involving partial dissociation of the



SCHEME 9. Metal chelate-catalyzed oxidation of ascorbic acid by dioxygen.

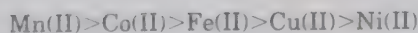
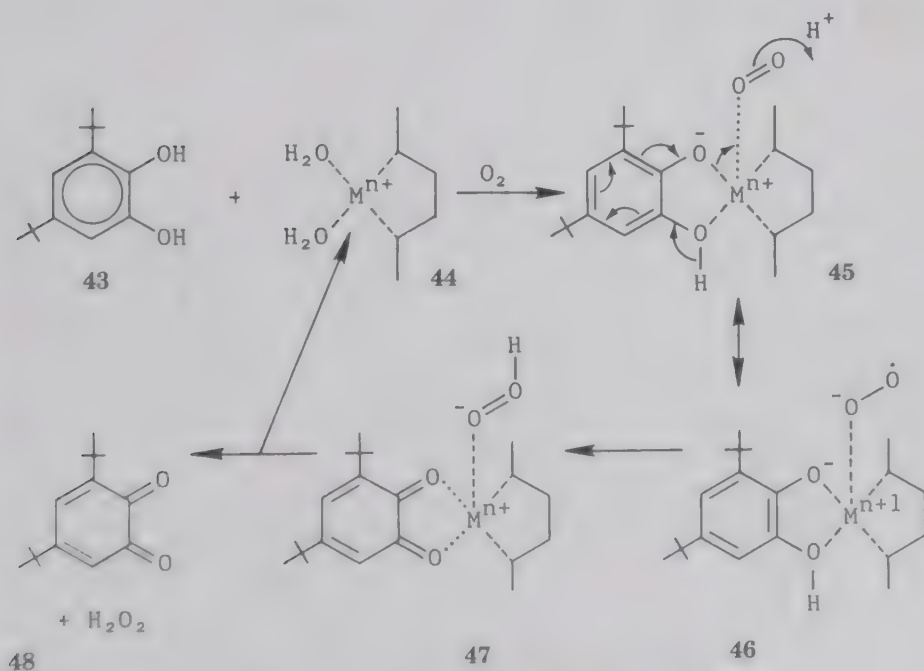
donor groups around the catalytic metal center is therefore entirely tentative.

The possibility that these metal chelate-catalyzed reactions occur through dissociation of the metal chelate to the free aquo metal ion which then undergoes the reaction scheme shown for catalysis by the aquo ions of Cu(II) and Fe(III) was investigated by calculating the concentrations of free aquo ions from available equilibrium data. Such calculations were not successful in reproducing the observed rates, and the reactions are therefore assumed to involve rate-determining steps involving the metal chelates as a whole. Thus, catalysis by each metal chelate is characterized by steric factors specific to that particular complex as well as by the energy required to carry out sufficient unfolding of the multidentate ligand to allow contact with the ascorbate anion.

The catalytic effects observed for various metal chelates involving Cu(II) and Fe(III) illustrate an important factor that must be considered in the preservation of ascorbic acid in foods. It is obvious that merely adding a chelating agent to suppress the catalytic effects of trace metal ions does not completely eliminate ascorbate oxidation by molecular oxygen derived from the air. The chelating agent merely slows the reactions but does not eliminate them. It should be possible, however, to estimate shelf life of foods preserved in this way by ac-

tually measuring the catalytic oxidation rates in the presence of various chelating agents and known trace levels of catalytic metal ions.

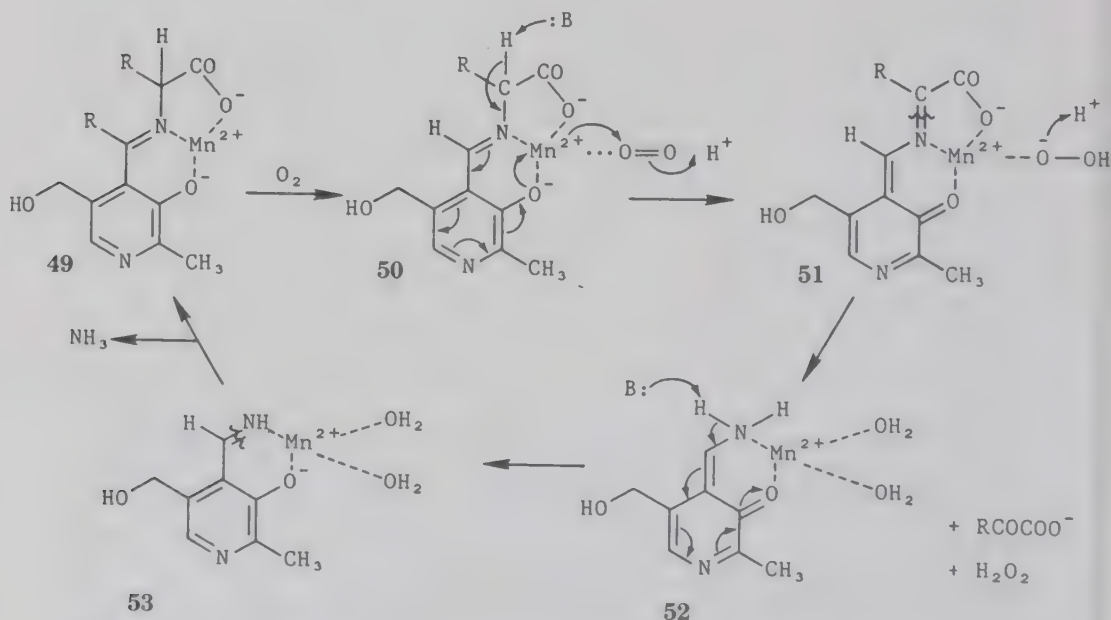
Oxidation of Catechols. The oxidation of catechols to the corresponding *o*-quinones by molecular oxygen is catalyzed by complexes of transition metal ions (Grinstead 1964; Tyson and Martell 1972). To prevent precipitation of the metal ion under the conditions employed, the catalytic metal ions were kept in solution as soluble complexes of halogenated catechols, which are resistant to oxidation by molecular oxygen (Tyson and Martell 1972). The reaction rates were found to exhibit first-order dependence on the concentrations of the catechol being oxidized, of the metal complex used as catalyst, and of molecular oxygen. Here also, the dependence of the observed rate on oxygen concentration indicates the possible formation of intermediate quarternary metal-ligand-substrate-dioxygen complexes in which the rate-determining electron transfer takes place. Hydrogen peroxide was found in the reaction mixture, but only in small amounts, indicating the possibility of disproportionation of hydrogen peroxide to oxygen and water through the catalase action of the metal catalyst employed. A typical reaction sequence (Scheme 10) is indicated by 43-48, repre-



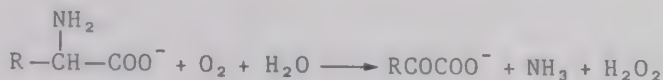
SCHEME 10. Metal chelate-catalyzed oxidation of catechols by dioxygen.

senting stepwise oxidation of ditertiary butylcatechol through single electron transfer steps. In the suggested mechanism the metal ion is represented as remaining coordinated to the carrier ligand and perhaps also to the substrate while undergoing its oxidation-reduction cycle. This is considered a good possibility in reaction systems of this type. The final product, *o*-quinone, is a very poor donor ligand and would be expected to release the metal ion, thus making it available for continuation of the catalytic cycle.

The Oxidation of Amino Acids. The oxidation of α -amino acids to the corresponding α -keto acids by molecular oxygen is catalyzed by pyridoxal and transition metal ions. In natural systems, such as the amine and diamine oxidases, Cu(II) seems to be the activating metal ion. In model systems several metal ions seem to activate the process, including those of Cu(II), Co(II), Fe(II), and Mn(II), the latter having the greatest activity (Hamilton and Revesz 1966; Haruta and Martell 1985). In this system (Scheme 11), the electron transfer step was suggested as occurring in a dioxygen complex formed from the metal chelate of the Schiff base of pyridoxal with the amino acid undergoing oxidation. In the reaction sequence illustrated by 49–53, electron transfer from the amino acid to dioxygen is indicated as occurring through a series of ionic-type two-electron transfer steps. This process



SCHEME 11. Metal ion- and pyridoxal-catalyzed oxidation of amino acids by dioxygen.

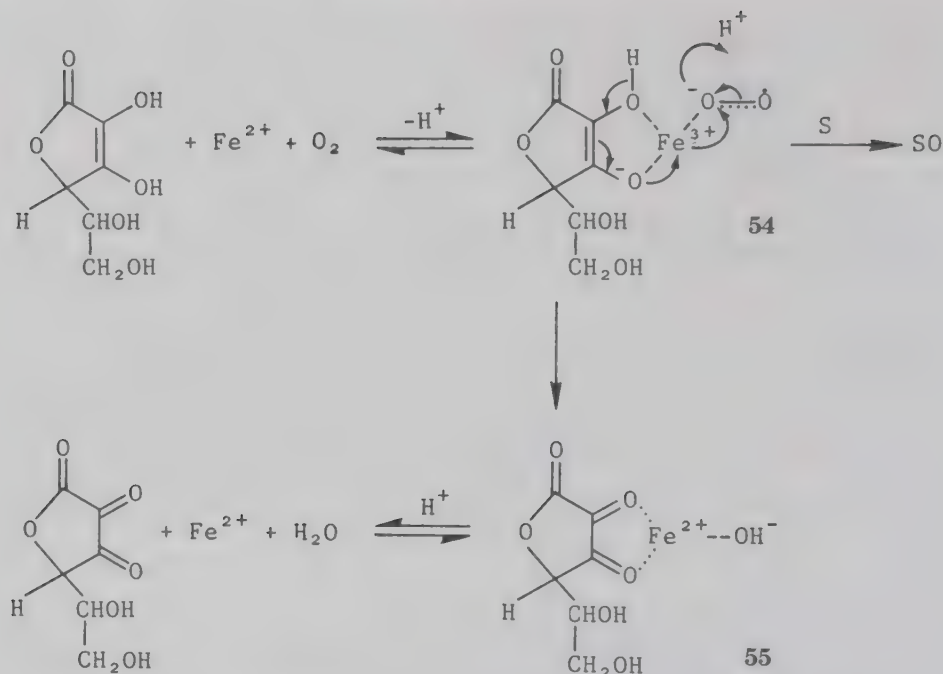
Overall reaction

is considered to be initiated by metal ion-catalyzed dissociation of the α -proton of the amino acid in a manner similar to the reaction that occurs in normal pyridoxal-catalyzed transamination reactions (Martell 1982). In the presence of dioxygen, however, the pyridoxal is not reduced to pyridoxamine, but is maintained in its oxidized form. The overall reaction may be concerted with simultaneous formation of the keto acid and release of pyridoxal, ammonia, and hydrogen peroxide. Although the reaction is indicated as occurring via a two-electron transfer process, it is considered possible or even probable that it consists of two successive single electron transfers.

Hydroxylation of Aromatic Compounds. Ferric or ferrous ions catalyze oxygen insertion into aromatic compounds in the presence of a ligand such as EDTA and a reducing agent such as ascorbic acid (Udenfriend *et al.* 1954; Brodie *et al.* 1954). Although free radicals had been considered to be the active species in this type of system, Hamilton (1969) noted that oxygen insertion occurs preferentially at the ortho and para positions relative to activating hydroxyl or methoxyl group on the aromatic ring and that free radicals therefore do not seem to be involved. On that basis, he suggested an ionic mechanism (Scheme 12) involving the formation of a ternary ascorbate-metal-dioxygen complex of the type indicated by 54. The reaction sequence shown suggests a concerted shift of electron pairs resulting in the insertion of an oxygen atom into the substrate simultaneously with two-electron reduction of ascorbate to dehydroascorbic acid. Of course, an alternate electron transfer process is possible involving two single electron transfer steps in place of the concerted mechanism shown. The proposed mechanism involving the formation of a metal-dioxygen complex intermediate is somewhat more satisfying than the free radical mechanisms previously suggested because it assigns a significant role to the metal ion, which is an essential catalyst in these hydroxylation systems.

Oxygen Insertion with Peroxide as the Oxidant

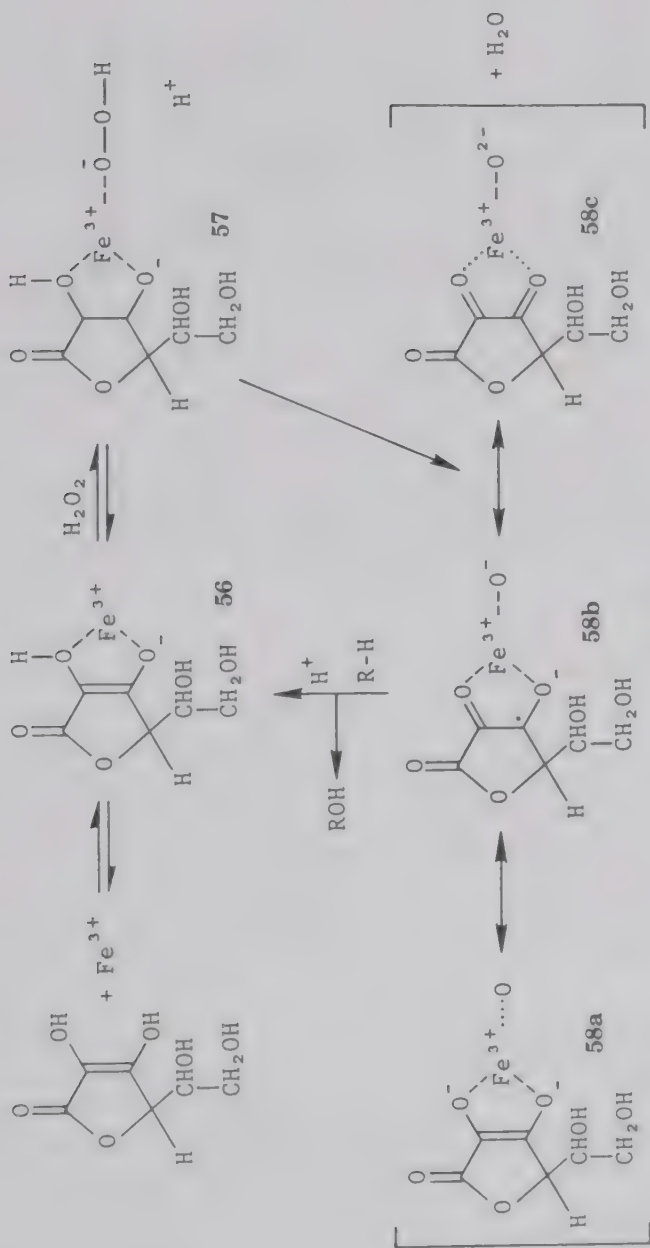
Organic compounds are hydroxylated by hydrogen peroxide in the presence of the iron(III) EDTA chelate and a suitable two-electron



SCHEME 12. Iron(II)-catalyzed oxygen insertion (Udenfriend's system).

donor such as ascorbic acid (Taqui Khan and Martell 1974; Grinstead 1960). The mechanism originally suggested involved the formation of hydroxide radical produced by the reaction of Fe(II) EDTA with hydrogen peroxide. The hydroxide radical was considered the reactive species involved in the insertion reaction while the ascorbic acid served the function of reducing the Fe(III) EDTA formed in the Fe(II) EDTA- H_2O_2 reaction back to the original Fe(II) EDTA to repeat the cycle.

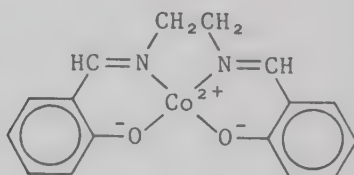
A fundamentally different mechanism (Scheme 13) was suggested by Hamilton and Revesz (1966) as a result of studies involving catechol as the two-electron reductant. The mechanism suggested involves the formation of a mixed catechol-Fe(III)- H_2O_2 complex which forms an active *o*-quinone-Fe(IV)-O intermediate. This intermediate, which is stabilized somewhat by delocalization of the electrons throughout the system, was considered to be the active hydroxylating species and to be similar to the intermediates proposed for the reactions of catalase and peroxidase enzymes. This interesting reaction mechanism is represented by **56**–**58**, with ascorbate as the two-electron reductant in this case. Insertion of the oxygen atom into an appropriate substrate results in regeneration of the original Fe(III) complex which then is again available for reaction with hydrogen peroxide to repeat the cycle.



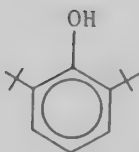
SCHEME 13

Oxidation Reactions with Dioxygen Complexes as Oxidants or as Intermediates

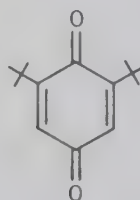
Oxidation of Phenols. The first report of the oxidation of phenols by dioxygen complexes was that of Van Dort and Geurson (1967) on the use of salcomine (**59**) [bis(salicylaldehyde)ethylenediiminecobalt(II)] as catalyst in the oxidation of 2,6-disubstituted phenols (**60**) with molecular oxygen. The formation of a dioxygen complex in this system is well known, as is the electron transfer reaction of the corresponding benzoquinone (**61**), involving oxygen insertion. The corresponding diphenylquinone (**62**), which requires oxidative dehydrogenation but not oxygen insertion, is also formed. This work was followed up by the extensive studies of Nishinaga and co-workers on the use of salcomine and similar oxygen-carrying complexes for the oxidation of hindered phenols (Matsuura *et al.* 1970; Nishinaga *et al.* 1974), 3-substituted indoles (Nishinaga 1975), and 3-hydroxyflavones (Nishinaga 1977). Perhaps the most significant contribution of the Nishinaga group (Nishinaga *et al.* 1981) is the isolation and identification of



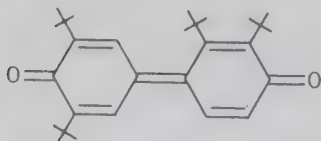
59 Salcomine



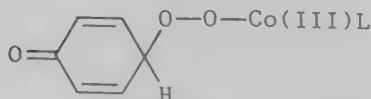
60 2,6-Ditertiarybutylphenol



61 2,6-Ditertiarybutyl-*p*-quinone



62 2,6,2',6'-Tetratertiarybutyl diphenylquinone



63 Quinolato-*p*-peroxocobalt(III) complex
(L = salicylaldehydiimine)

SCHEME 14

the quinolato-*p*-peroxocobalt(III) complex (63) as an intermediate in the conversion of the phenol to the corresponding quinone. This intermediate is the site of attack by the coordinated dioxygen on the organic molecule, thus demonstrating the role of dioxygen complexes in oxygen insertion reactions.

Another interesting example of catalysis by salcomine is the oxidation of ascorbic acid by molecular oxygen. The reaction is first order in salcomine and probably proceeds through successive one-electron transfers from the reductant to molecular oxygen through the Co(II)-dioxygen complex. A ternary complex involving the combination of the salcomine-dioxygen complex with ascorbic acid was proposed as the intermediate in which the rate-determining electron transfer occurs (Nishinaga *et al.* 1977).

Oxidative Dehydrogenation of Coordinated Ligands in Dioxygen Complexes. The oxidative attack of a coordinated ligand by coordinated oxygen in metal-dioxygen complexes represents still another kind of metal ion activation of molecular oxygen. One of the first examples of this type of reaction is the determination of the site of oxidative attack of coordinated glycylglycine and other dipeptides in the cobalt-dioxygen complexes formed with these peptides as ligands. It was found (Harris *et al.* 1977; Harris and Martell 1980A,B) that the coordinated μ -peroxo group in these complexes undergoes a two-electron reduction with simultaneous oxidation of the N-terminal amino group of a coordinated dipeptide to the corresponding imine. This type of reaction takes place readily at room temperature and could be a key reaction in the oxidative degradation of peptides in the presence of metal ions such as Co(II) and Fe(II) that readily form dioxygen complexes.

Another example of the oxidative attack of a coordinated ligand by dioxygen complexes is the oxidative dehydrogenation of aliphatic polyamines. Recent studies of this reaction (Raleigh and Martell 1984) show that it takes place readily under mild conditions, but is sensitive to the molecular environment of the coordinated polyamine. The reaction has been observed to occur with the formation of unsaturated imine groups when an aromatic substituent is present in a position favorable for conjugation with the imine group generated in the oxidation process. Also, it has been found that conformations of the polyamine in the coordination sphere of the cobalt(III) must be favorable for generation of the conjugated double bond; otherwise oxidative attack on the coordinated polyamine does not occur or occurs with much greater difficulty.

ACKNOWLEDGMENT

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Free Radical Chemistry of Natural Products

P. Neta¹ and M.G. Simic²

INTRODUCTION

Free radicals in plant and animal products are far from being thoroughly investigated. Their direct measurements are often lacking and their existence has been usually inferred from either observed products or oxygen depletion studies (Simic and Karel 1980). In both approaches, the deductions are based on comparison with model systems, where free radicals can be observed directly (see, e.g., Hoffman 1981) by electron spin resonance (ESR), spectrophotometry, or some other physicochemical techniques, under strictly controlled conditions. It should be pointed out, however, that only the ESR technique (Sevilla 1981) gives an unequivocal detection of free radicals because it is based on the measurements of unpaired electrons.

In model systems, free radicals can be conveniently studied by pulse radiolysis (Hoffman 1981) and laser photolysis (Bensasson *et al.* 1983), the two techniques most widely used. Both ionizing radiation (X rays, high-energy electrons, etc.) and light (visible and ultraviolet) are used to generate free radicals. The pulse mode in which radiation and light are delivered also allows kinetic measurements of free radical pro-

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cesses. Since in both cases the pulse length is normally a few nanoseconds and can be as short as a few picoseconds, some very fast free radical processes can be studied.

Free radicals in natural products are produced in normal physiological reactions, e.g., in synthesis of sterols from arachidonic acid (Marshall *et al.* 1984), on oxidation of RSH compounds (Asmus 1983; Willson 1983), or on one-electron reduction of oxygen either directly or enzymatically (Bielski 1983A).

Stationary state concentrations of free radicals induced by these processes are extremely low and very difficult to measure. Much higher yields of free radicals are generated by exogenous processes such as light, radiation, and heat. Light is a normal companion of natural products and is responsible for photosensitized generation of free radicals via intermediary excited states and singlet oxygen (Foote 1984). Light, as fluorescent light in supermarkets, could be deleterious to numerous processed foods, and more attention should be paid to eliminating unnecessary exposure. Light-induced free radical processes are dealt with in this volume by Foote (Chapter 2), and no further considerations will be given here to these reactions.

Radiation has lately become acceptable as a food preservation process (Josephson and Peterson 1983). Free radicals (from ions and excited states) are generated in fairly large quantities. For example, at 1 Mrad, under normal conditions (room temperature, hydrated foods), 3–6 mmol of free radicals are produced per 1 kg of food. Radiation chemistry of foods will not be treated in this chapter; however, radiation chemistry of some simple chemical systems will be used as a model for free radical reactions in general.

Heat in food processing is an important factor for preservation of foods and, more importantly, it can induce highly desirable flavors (see Chapter 1, this volume). As far as free radical reactions are concerned, heat has a dual role. It accelerates free radical reactions in autoxidation (Porter 1980). For example, it takes only a few hours at 70°C to have the same extent of autoxidation which at room temperature requires months. Such effects are easily followed from the rate measurements of oxygen depletion (Porter 1980). The second, more important effect of heat on food is at high temperatures, such as temperatures of frying 200°–300°C, where pyrolysis of food components take place with concomitant formation of free radicals (Nawar 1983). The understanding of such processes is still limited and will not be dealt with here.

Our major attention will be focused on free radicals in natural products associated with autoxidation processes, mainly oxygen radicals, for a number of reasons. Autoxidation is the main contributor to free radical processes in foods. It is also one of the major problems in

food processing and preservation, although not always recognized as such.

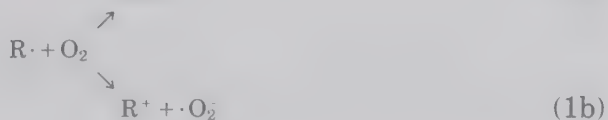
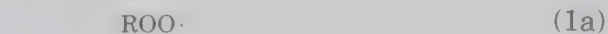
Oxygen-centered free radicals in natural products can be subdivided into the following oxygen-centered classes

$\text{ROO}\cdot$	Peroxyl radicals
$\text{RO}\cdot$	Alkoxy radicals
$\cdot\text{O}_2^-$	Superoxide radical
$\cdot\text{OH}$	Hydroxyl radical

(Oxygen-centered radicals will be referred to as oxyl, e.g., peroxyl, alkoxy, although certain texts use the terms "peroxy" and "alkoxy.") Kinetics and mechanics of these free radicals and their interrelationships will be briefly discussed below.

PEROXYL RADICALS

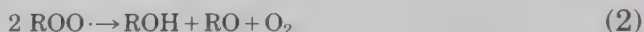
Initial formation of free radicals in food has been a subject of much debate and there is still no consensus among experts in the field. There are various possibilities, however. Initial free radicals can be formed by photosensitization (Foote 1984), singlet oxygen (Foote 1984), reaction of oxygen with reductive metabolites (Bielski 1983A) (e.g., RSH, NADH), and some enzymatic processes (Bielski 1983A). It is also plausible that some food additives can induce formation of free radicals, for example, interaction of nitrite with ascorbate. Whatever the initial reactions are, most of those free radicals will react with oxygen, which is present in foods, either by addition or by oxidation (Simic 1981).



Reactions of oxygen with free radicals are extremely fast (Simic 1981), $k \sim 10^9 \text{ M}^{-1} \text{ sec}^{-1}$. Hence, the lifetime of free radicals will be very short. Which way the pathway of reaction 1 will go depends on the nature of these free radicals and the polarity of the medium. Addition of oxygen (1a) is prevalent in lipids, while electron transfer (1b) takes place predominantly in aqueous solutions at higher pHs. Sometimes the peroxyl radicals produced by reaction 1a are unstable and decay

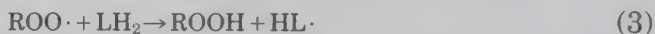
by elimination of O_2^- , giving an overall effect of reaction 1b having taken place.

Isolated peroxy radicals disappear in a reaction with each other to give hydroxy and carbonyl derivatives:



The rate of the above reaction is very high, $k \sim 10^7 - 10^9 M^{-1} \text{ sec}^{-1}$, and it may go through an intermediary unstable tetroxide.

The damaging character of peroxy radicals stems from their ability to abstract hydrogen from weak hydrogen bonds (Ingold 1969; Howard 1972).



Particularly weak C—H bonds are those adjacent to and between two double bonds as found in various unsaturated lipid molecules. Bond energies in these molecules can be as low as 75–85 kcal/mol (Yukawa 1965), while the $ROO-H$ bond energy is estimated to be about 90 kcal/mol (Benson and Shaw 1968).

The $HL\cdot$ radical would react with oxygen to give an $HLOO\cdot$ peroxy radical, which would react with LH_2 molecules and propagate a chain.



Consequently, one initial radical can damage a few hundred lipid molecules.

The damage of the peroxy radical does not end there. The resultant hydroperoxide can be readily reduced by metabolic reductants, and also by iron which is quite abundant in cells and foods.

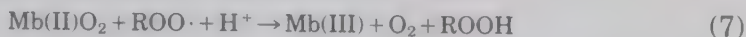


The oxy radicals $HLO\cdot$ are very reactive and can generate a new family of $R\cdot$ radicals and new sets of chain reactions. Sometimes only a small amount of iron is sufficient to propagate the branching of chains because reductive food components can regenerate $Fe(II)$. For example,



Complexing of $Fe(III)$ with certain ligands would reduce the redox potential of the complex, which in turn would prevent the $Fe(II)$ regeneration process.

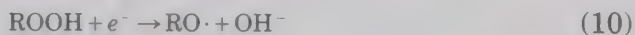
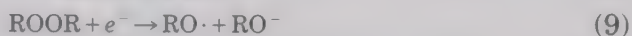
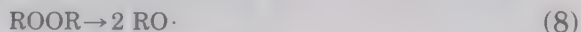
The peroxy radicals can do other kinds of biochemical damage. For example, the browning of red meats can be effected by water-soluble peroxy radicals (Simic 1983).



The reaction is not very fast ($k \sim 10^3 M^{-1} \text{ sec}^{-1}$), but sufficiently fast to be considered an important factor in autoxidation of ground meats.

ALKOXYL RADICALS

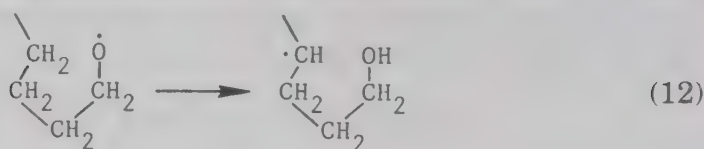
Alkoxy radicals are produced from peroxides or hydroperoxides by photolysis or by one-electron reduction (e.g., with metal ions) (Howard 1972; Gilbert *et al.* 1976, 1981; Neta *et al.* 1984; Paul *et al.* 1978):



Alkoxy radicals in general are more reactive and shorter lived than peroxy radicals, and their mode of reaction is often different. Alkoxy radicals react with organic compounds by H abstraction or by addition to double bonds more rapidly than peroxy, but they are less likely to engage in redox reactions. More importantly, alkoxy radicals may undergo unimolecular rearrangements or decomposition, often preferentially to reaction with other molecules. The behavior of alkoxy is also different in some respect from that of aryloxy, which is discussed under antioxidants in Chapter 7, this volume.

Hydrogen abstraction by alkoxy was found to take place generally with rate constants of 10^5 – $10^8 M^{-1} \text{ sec}^{-1}$ (see, e.g., Neta *et al.* 1984; Paul *et al.* 1978), the lower range for saturated aliphatics and the highest for very activated hydrogens, e.g., in phenols. Addition to double bonds occurs with moderate rate constants, generally 10^6 – $10^7 M^{-1} \text{ sec}^{-1}$. These rate constants are not sensitive to solvent composition, except when the H to be abstracted becomes highly hydrogen bonded (e.g., phenols in water) and thus more difficult to abstract. In the foregoing reactions, alkoxy radicals generally react more slowly and more selectively than the OH radical (Neta *et al.* 1984).

The unique reactions of alkoxy are the intramolecular processes, mainly the 1,2- and 1,5-hydrogen shifts (reactions 11 and 12) in primary and secondary radicals and the β -scission (13) in tertiary radicals (Gilbert *et al.* 1976, 1977, 1981; Neta *et al.* 1984; Paul *et al.* 1978; Baignee *et al.* 1983).

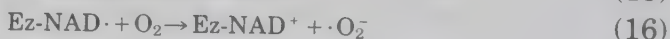
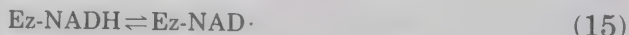
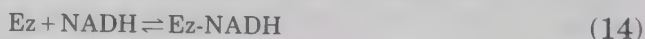


These reactions are generally very rapid (10^6 – 10^7 sec $^{-1}$) and their rates are solvent dependent. They compete effectively with the bimolecular reactions of alkoxyl and result in the conversion of these radicals into the less reactive alkyl radicals (which in the presence of oxygen form the corresponding peroxy radicals) or into the reducing α -hydroxyalkyl radicals.

SUPEROXIDE AND HYDROPEROXYL RADICALS

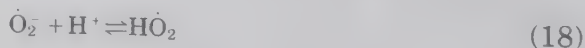
As we have indicated above, $\cdot\text{O}_2^-$ can be formed by oxidation of reductive metabolites (such as RS^-) and free radicals (reaction 1b) as well as by decomposition of peroxy radicals.

Another interesting reaction is of an enzymatic nature and initiated by faulty occurrence of a one-electron redox reaction associated with NADH:



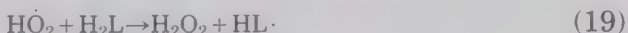
Lactate dehydrogenase, for example, can yield chain lengths as high as 70 (Bielski 1983).

The chemistry of the superoxide radical, $\dot{\text{O}}_2^-$, has been studied and reviewed by numerous authors (see, e.g., Sawyer and Valentine 1981; Sawyer and Nanni 1981; Frimer 1982) and has been the subject of several meetings (International Conferences on Oxygen Radicals, see, e.g., Marshall *et al.* 1984; Sawyer and Nanni 1981; International Conferences on Superoxide Dismutase, see, e.g., Cohen and Greenwald 1983). In organic aprotic media $\dot{\text{O}}_2^-$ is stable and may engage in redox reactions, both as an oxidant (of certain metal ions) and as a reductant (of quinones, metal ions, etc.), or may act as a strong base to abstract a proton and thus facilitate subsequent oxidation. In aqueous media $\dot{\text{O}}_2^-$ is fairly unreactive and is a very weak base. The equilibrium



has a pK_a of 4.8. The acid form $\dot{H}O_2$ is much more reactive than $\dot{O}_2^-$ as an oxidant, and many of the reactions ascribed to $\dot{O}_2^-$ appear to be due to $\dot{H}O_2$.

Superoxide radical has been implicated in various damaging processes of a biological and biochemical nature (Fridovich 1972; Cohen and Greenwald 1983). However, conclusive evidence for damaging reactions has been missing. More recently, Bielski (1983B) has pointed out that $\dot{H}O_2$ radicals as a stronger oxidant could be much more reactive than $\cdot O_2^-$ and that could perhaps initiate autoxidation in membranes.



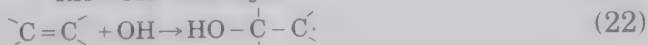
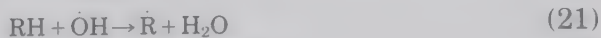
If that were the case, then the conditions which favor $\cdot O_2^-$ formation by many biochemical processes may contribute also to spoilage of fresh vegetables and fruits.

HYDROXYL RADICAL

The OH radical is produced by irradiation of water or by photolysis of hydrogen peroxide. In the natural environment OH is believed to be formed from H_2O_2 by a Fenton-type reaction with reduced metal ions (Cohen and Greenwald 1983):



This radical is the most reactive of all oxygen radicals (Farhataziz and Ross 1977). It can practically attack any organic compound and either abstract hydrogen or add to double bonds, with rate constants of 10^8 – $10^{10} M^{-1} sec^{-1}$, i.e., very often at diffusion controlled rates.

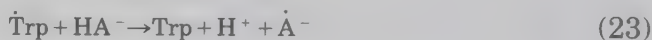


Therefore, $\dot{O}H$ can only react and cause damage at the site of its formation.

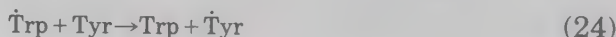
OTHER RADICALS

Other types of radicals may be formed in food by irradiation or by "natural" processes, either from food components or from food additives. We will mention here some representative examples.

Aliphatic amino acids undergo H abstraction to form alkyl-type radicals which react rapidly with oxygen to give either peroxy radicals or $\dot{O}_2^-$, depending on their structure (see, e.g., Dinur-Abramovitch and Rabani 1976; Bothe *et al.* 1978). Tyrosine and tryptophan are easily oxidized to form radicals which may engage in further electron transfer. For example, both of these radicals can oxidize vitamins E and C to their respective radicals.



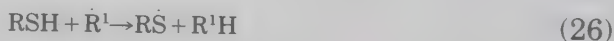
It is also interesting to note that the radical produced from tryptophan was found to oxidize tyrosine by an intramolecular electron transfer within proteins, so that an initial attack on tryptophan may actually end up as damage to a tyrosine (Butler *et al.* 1982).



Sulfur-containing amino acids and peptides also form radicals very easily, for example, by adding an electron to cystine,

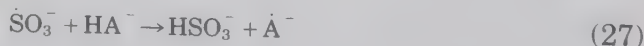


or by abstracting H from cysteine,

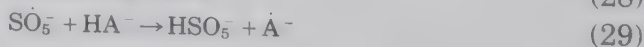
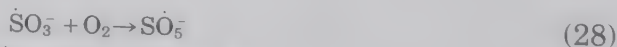


The latter reaction is viewed as a repair process whereby $\dot{\text{R}}$ is prevented from reacting with O_2 and propagating damage (Wilson 1983; Asmus 1983).

The food additive sulfite or bisulfite can be easily oxidized to form the $\dot{\text{SO}}_3^-$ radicals. These radicals were found (Huie and Neta 1984) to oxidize ascorbate



and tocopherol, and may attack a variety of other compounds. More importantly, $\dot{\text{SO}}_3^-$ reacts very rapidly with O_2 to form a peroxy-type radical, SO_5^- , which is more reactive and a stronger oxidant than $\dot{\text{S}}\text{O}_3^-$.



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Mechanism of Fatty Acid and Phospholipid Autoxidation

*Ned A. Porter*¹

Autoxidation, the reaction of molecular oxygen and hydrocarbons, is one of the most general reactions in organic chemistry. The reaction of free radicals with oxygen is extremely rapid, and many mechanisms for initiation of free radical reactions have been described. The weak link with regard to autoxidation is those lipids which contain multiple double-bonded systems. Polyunsaturated fatty acids and phospholipids, for example, are particularly prone to autoxidation, and the susceptibility of these compounds to autoxidation has been known for decades. There are many examples which suggest that autoxidation plays a crucial role in the development of flavors and aromas, and this is the focus of many of the chapters in this volume.

Hydroperoxide products are recognized as the first-formed major products in autoxidation, and linoleic acid was determined years ago to form hydroperoxide products with substitution of oxygen at positions 9 and 13 of the fatty acid chain (Fig. 1). The mechanism generally accepted for fatty acid autoxidation is shown in Scheme 1. Initiator radicals react with the substrate to generate a radical, $L\cdot$, formed by an H atom transfer. The two propagation steps involve addition of oxygen to $L\cdot$ to give a peroxy radical, $LOO\cdot$, a reaction known to proceed with a rate nearly diffusion controlled, and a slow step in which

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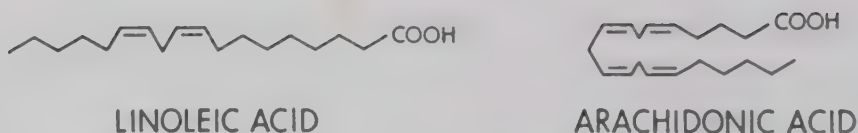
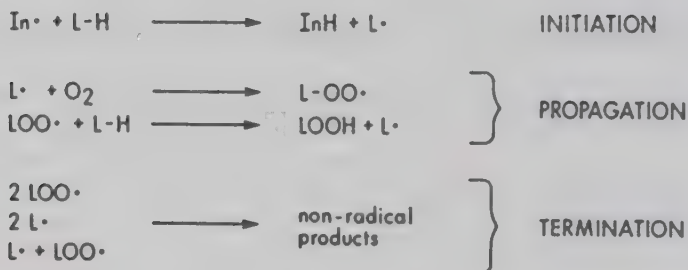


FIG. 1.



SCHEME 1

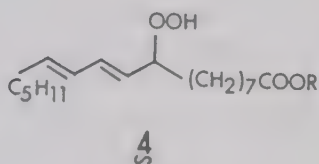
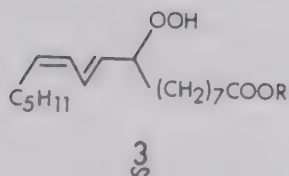
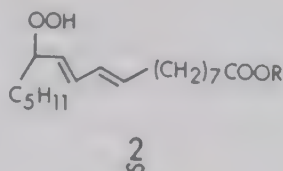
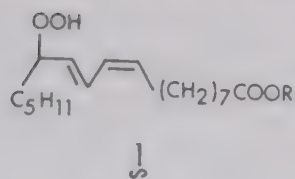
the peroxy radical ($\text{LOO}^\bullet$) abstracts hydrogen from L-H to give the product hydroperoxide and another $\text{L}^\bullet$.

Formation of 9- and 13- substituted hydroperoxides from linoleic acid may be understood by the fact that linoleic acid gives rise to $\text{L}^\bullet$ which is delocalized over five carbon centers. Thus, the H atom removed from the acid is located at position 11 and the carbon radical is delocalized between positions 9 and 13 of the fatty acid. Addition of oxygen to the termini of the delocalized radical gives rise to peroxy radicals at positions 9 and 13, which are ultimately converted to the hydroperoxides.

Phospholipid autoxidation proceeds by the same mechanism as the fatty acid process. The primary products formed from autoxidation of linoleic acid, its methyl ester or its esters of glycerophospholipids, are the hydroperoxides substituted at the 9 and 13 positions. Thus, autoxidation of free fatty acids in bulk, methyl esters in solution, or phospholipids in aqueous emulsion follow essentially the same course.

In fact, four major products, 1-4, are formed from linoleate autoxidation. Two of the products, 1 and 2, have oxygen substitution at the 13 position and two have substitution at C-9. The products differ by virtue of the fact that they have different stereochemistry about the conjugated diene. Thus, compounds 1 and 3 have trans,cis conjugated diene, while 2 and 4 are trans,trans compounds.

The relative amounts of products 1-4 formed in autoxidation depend on the medium of oxidation. In general, the amount of products formed at the 9 and the 13 positions is equivalent. Thus, in general, $1+2=3+4$, regardless of the conditions of oxidation. The ratio of



products having trans,cis diene stereochemistry vs those having the trans,trans structure does vary dramatically with concentration, temperature, and medium of oxidation, however. For example, autoxidation of 0.24 *M* linoleic acid in benzene or chlorobenzene at 30°C gives a ratio of $1 + 3/2 + 4$ equal to 0.24. Under the same conditions, 1 *M* linoleate gives a product ratio of 0.56. Another example is even more dramatic. Autoxidation of 0.24 *M* linoleic acid in 1,4-cyclohexadiene, an excellent H atom donor, gives a $1 + 3/2 + 4$ product ratio of over 20. Thus, the distribution of products depends dramatically on the medium of oxidation. In benzene, a poor H atom donor, trans,trans products are the dominant products. In 1,4-cyclohexadiene, the products are almost exclusively trans,cis. A detailed discussion of this mechanism has been presented elsewhere (Porter 1984). For purposes of this discussion, it is important to note that the mechanism for formation of the trans,cis and trans,trans products involves a reversible oxygen addition to intermediate lipid radicals. Thus, addition of oxygen to the radical $L^\cdot$ initially formed from linoleic acid gives a trans,cis diene peroxy radical which will abstract hydrogen to give the trans,cis diene hydroperoxide product (Fig. 2). Loss of oxygen from the trans,cis peroxy radical ($t,c\text{-LOO}^\cdot$) can generate $L^\cdot$ or an isomeric radical which can lead ultimately to a $t,t\text{-OOH}$ product. The crucial competition, then,

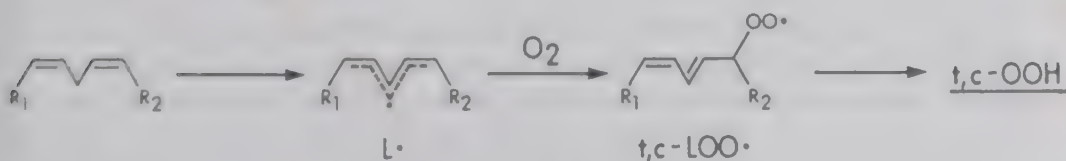


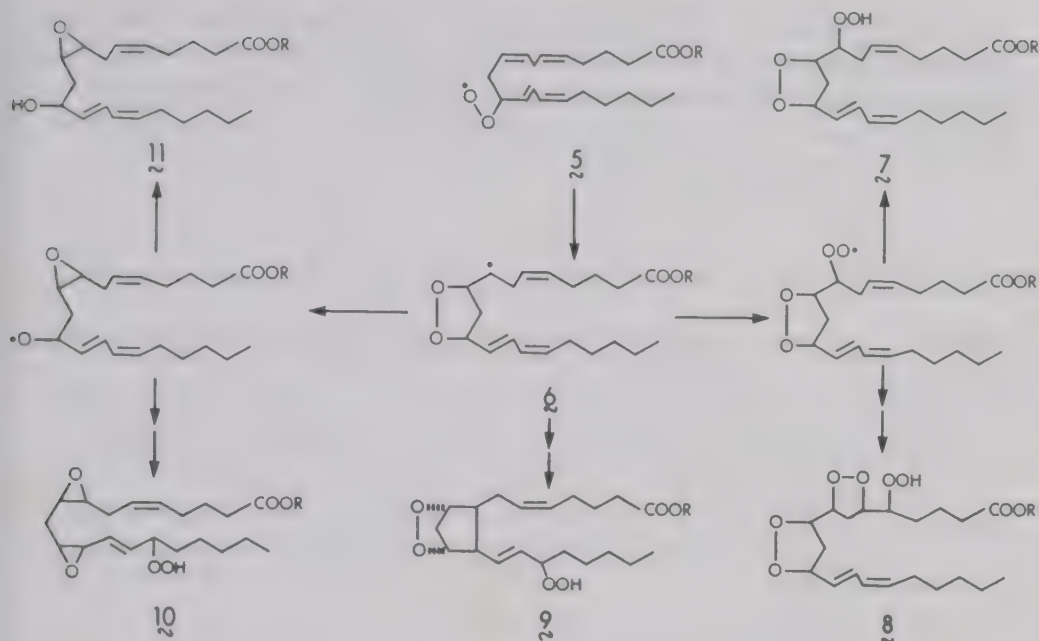
FIG. 2.

is the competition between conversion of the *t,c*-LO $\cdot$ intermediate to trans,cis hydroperoxide product and loss of oxygen from the peroxy radical giving an isomerized L $\cdot$ and ultimately *t,t*-OOH. Good hydrogen atom donors promote the transfer of H to the original LOO $\cdot$ and favor formation of trans,cis products. As noted earlier, if linoleic acid is autoxidized in benzene, a poor H atom donor, the trans,trans diene products comprise over 80% of the product mixture. If, on the other hand, the autoxidation is carried out in 1,4-cyclohexadiene, an excellent H atom donor, the trans,cis product is over 97% of the product mixture. The solvent thus determines the amount of the products formed.

Autoxidation of linoleate phospholipids follows a course similar to autoxidation in solution. If good hydrogen atom donors are available to linoleate peroxy radicals generated on the phospholipid, the major products are trans,cis dienes. If no good H atom donors are present, the major products are trans,trans dienes. This effect is observed for phospholipid autoxidation in aqueous emulsion. Autoxidation of dilauroic glycerophosphatidylcholines (DLGPC) at 37°C gives a *t,c/t,t* product ratio of 1.3. Addition of α -tocopherol to the aqueous emulsion of DLGPC gives a product ratio of over 70, the trans,cis product being favored. In aqueous emulsion then, the good donor α -tocopherol traps peroxy radicals before loss of oxygen can occur.

Recently, two potential inhibitors of autoxidation, vitamin C and uric acid, have received increased attention. Both of these water-soluble compounds have been suggested to play a role as an inhibitor of autoxidation in lipid bilayers. We have applied our product distribution test to determine the efficacy of vitamin C and urate as inhibitors of autoxidation in lipid bilayers. Autoxidation of DLGPC emulsions with 10^{-3} M vitamin C increased the *t,c/t,t* product ratio from 1.3 (no added vitamin C) to over 3.5 (10^{-3} M vitamin C). This increase in product ratio indicates that ascorbate in the aqueous phase can influence product distribution of autoxidation in the hydrophobic bilayer. The effect, however, is small when compared to the effect observed with α -tocopherol (see above). In the case of the fat-soluble α -tocopherol, the hydrogen atom donor is localized in the interior of the lipid bilayer. The tocopherol is thus localized where autoxidation occurs, and at low concentrations its effect is felt dramatically. Vitamin C has a much reduced effect with very high concentrations required to produce any noticeable change in product ratio. No effect was noted at any concentration of urate used. Our product ratio test thus suggests that uric acid is ineffective as an antioxidant for reactions carried out in the lipid interior.

A final aspect of phospholipid autoxidation to be considered is the



SCHEME 2

broad spectrum of products observed for tri- and tetraene lipids. We have examined products derived from polyene systems and have determined that peroxy radical cyclization is an important pathway for tri- and tetraene fatty acids and phospholipids. In particular, peroxy radicals generated at positions where 5-exo cyclization may occur are prone to undergo this reaction. Thus, peroxy radicals such as those described in Scheme 2 cyclize to give a host of products. Peroxy radical 5 can undergo 5-exo cyclization to give carbon radical 6 that has multiple pathways available. Among these multiple pathways are oxygen entrapment (Porter *et al.* 1981) giving monocyclic peroxide (7) and serial cyclic product (Porter and Khan 1982) (8). An alternate pathway to oxygen addition is carbon cyclization (Porter *et al.* 1984) to the endoperoxide (9) and a third competing pathway for 6 is S_H radical attack (Porter and Nixon 1978) on the peroxy, giving ultimately epoxides 10 and 17. The mechanistic pathway for autoxidation of polyenes is thus extremely complex. The scheme presented here shows only one possible peroxy radical of the six possible trans,cis peroxy radicals that form from the tetraene fatty acid, arachidonic acid. It should further be noted that stereoisomers of each of the products 7–11 exist, further complicating the product mixture. Four diastereomers of 7 are formed, for example, while multiple stereoisomers

exist for each of the other products 8–11. The autoxidation of polyunsaturated fatty acids, esters, and phospholipids thus gives rise to a complex mixture of products that includes acyclic hydroperoxides, cyclic peroxides, and epoxides. The mechanisms leading to these products are reasonably well understood.

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Thermal and Radiolytic Decomposition of Lipids

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Radiation preservation of food is a relatively new process, the advantages of which have been recognized for many years. Its practical application, however, has been hindered by regulatory, technical, and economic problems. In the past few years a phenomenal progress toward the solution of these difficulties has taken place. In 1980, the joint Food and Agriculture Organization/World Health Organization/International Atomic Energy Agency Expert Committee on wholesomeness of irradiated food concluded that "the irradiation of any food commodity up to an overall average dose of 10 kilogray (kGy) (1 Mrad) causes no toxicological hazard and hence toxicological testing of foods so treated is no longer required." In February of 1984, the U.S. Food and Drug Administration proposed regulations to permit the treatment by ionizing radiation to inhibit growth and maturation of fruits and vegetables, at doses up to 1 kGy (100 krad), and to control infestation of spices at doses not exceeding 30 kGy (3 Mrad). Indeed, a number of irradiated foods are already on the market in several countries, and there is every reason to believe that in the near future, this treatment will become a significant process in food technology.

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Food irradiation has the same objective as heat processing, i.e., prolongation of shelf life. However, while the use of heat has been used since prehistoric times and is therefore relatively unquestioned, irradiation is new and requires rigorous examination. Understandably, comparison of the two treatments assumes special importance. It has now become clear that relying solely on animal feeding experiments for assessing the safety of individual food items under varying processing conditions is too costly, lengthy, and often unworkable. Thus, a comparative understanding of the chemistry involved, the products formed, and their biological effects for heat and radiation processing can be of great value to both processors and regulatory bodies.

In this chapter, the chemical changes resulting in food lipids by the two treatments are examined from the standpoints of the qualitative and quantitative patterns of the decomposition products formed and the mechanisms of their formation.

EXTENT OF DECOMPOSITION

When a lipid is exposed to heat or ionizing radiation, a certain amount of the substrate undergoes decomposition or chemical change, giving rise to new products. Of these, a relatively small portion consists of numerous volatile compounds of smaller molecular weight than the substrate. It is the nonvolatile products, however, which represent the bulk of the decomposition. These compounds consist of a mixture of higher molecular weight derivatives, dimers, or polymers. As will be seen, several similarities can be observed between the qualitative pattern of radiolytic products and those obtained by heat. However, the generalization may be made that heat treatment at temperatures normally used in food processing operations causes more overall decomposition than irradiation at the doses required for deinfestation, pasteurization, or even sterilization.

For example, when 1 g of ethyl linoleate was oxidized at 180°C (a typical frying temperature) for 1 hr, the volatiles produced amounted to 27%, the nonvolatiles amounted to 36%, whereas 65% of the substrate remained unchanged. By comparison, irradiation of ethyl linoleate under vacuum at 250 kGy (approximately five times the sterilization dose) produced 28% volatiles, 23% nonvolatiles, with 74% of the substrate unchanged (Sheu and Nawar 1984).

Although the total amount of volatiles produced by either heat or irradiation is relatively small, a fairly complete analysis of these compounds is now feasible by gas chromatographic and mass spectrometric techniques. Indeed, much insight into both the processes of

radiolysis and thermal oxidation has been obtained through volatile product analyses. Furthermore, many of these components are known to be of great flavor importance.

On the other hand, a detailed study of the more quantitatively significant nonvolatile products has been more challenging, the major difficulty being one of resolution. The increasingly fast rate of innovations in chromatographic techniques should contribute very significantly to our ability to investigate this important group of compounds.

In the following discussion, product analysis and mechanisms for each of the two processes will be discussed separately. Some comparative patterns of radiolysis and thermal degradation of lipids will then be given.

RADIOLYTIC REACTIONS

Products

Hydrogen, CO₂, CO, short-chain hydrocarbons, and water were reported in the early work on radiolysis of carboxylic acids by Shepard and Burton (1946), Burton (1949), and Newton (1957). Extensive qualitative and quantitative product analyses were conducted in the author's laboratory on a number of saturated and unsaturated fatty acids and their esters as follows: simple triacylglycerols with the component fatty acids C-4 to C-18; triacylglycerols containing the C-16:1, C-18:1, C-18:2, and C-18:3 acids; and the natural fats from beef, pork, mackerel, corn, soybean, olive, safflower, and cottonseed (Dubravcic and Nawar 1968, 1969; Champagne and Nawar 1969; Kavalam and Nawar 1969; LeTellier and Nawar, 1972A, B, 1974). More recently, Vajdi and co-workers reported the identification of higher molecular weight radiolytic products from palmitate, oleate, and beef fat (Vajdi and Nawar 1979; Vajdi *et al.* 1983).

It is now clear that the major compounds produced when a saturated fatty acid is exposed to ionizing radiation in the absence of oxygen are H₂, H₂O, CO₂, CO, a series of hydrocarbons (*n*-alkanes and 1-alkenes), a C_{*n*} aldehyde (*n* being the number of carbon atoms), a symmetric ketone, and α,α' -dimeric products. Of the hydrocarbon series, the hydrocarbon with one carbon less than the fatty acid is formed in the greatest quantity. The production of H₂ increases with an increase in molecular weight of the fatty acid. However, the amounts of both the C_{*n*-1} alkane and CO₂ are inversely proportional to fatty acid chain length (Wu and Howton 1974). In general, the pattern of radiolytic products from unsaturated fatty acids is similar to that of the

TABLE 6.1. RADIOLYTIC PRODUCTS OF TRIACYLGLYCEROLS

A. Breakdown products of smaller MW than substrate		
CO ₂ , CO, H ₂	Propanediol diesters	Carboxylic acids
<i>n</i> -Alkanes	Propenediol diesters	2-Alkylcyclobutanones
1-Alkenes	Oxopropanediol diesters	Monacylglycerols
Alkadienes	Aldehydes	Diacylglycerols
Alkynes	Ketones	Triacylglycerols
Esters	Lactones	Ethenediol diesters
B. Adduct Products		
Glyceryl ether diesters		
Propanedioldiester dimers		
Propanedioldiester-triacylglycerol adducts		
Triacylglycerol dimers		

saturated analogs, with the presence of double bonds in the substrate reflected in the compounds produced. In addition, significant amounts of dimeric or polymeric acids, cross-linked at carbon atoms adjacent to the double bonds, are formed.

The radiolytic pattern of products typically produced from each triacylglycerol is summarized in Table 6.1. It includes the same compounds arising from their component fatty acids and, in addition, other products of higher molecular weight reflecting the contribution of the glycerol moiety. Typical quantitative data are given in Table 6.2 for the radiolysis of the tricaproin. The major radiolytic product is the component fatty acid, the hydrocarbon produced in the greatest quantity is the C_{*n*-1} alkane, whereas the most abundant glyceryl-derived residues are the propanediol diesters. Not shown in Table 6.2 are the recombination and dimeric products. Quantitative analysis of these has been far more challenging.

TABLE 6.2. CONCENTRATION ($\mu\text{mol}/100\text{ g}$) OF SOME COMPOUNDS IDENTIFIED IN TRICAPROIN IRRADIATED AT 60 kGy AND 25°C

Methane	15	Methyl hexanoate	39
Ethane	20	Hexanoic acid	1220
Ethene	14	2-Oxopropyl hexanoate	25
Propane	12	6-Undecanone	70
Propene	4	2-Oxoheptyl hexanoate	30
Butane	10	1,2-Propanediol dicaproate	324
1-Butene	39	1,3-Propanediol dicaproate	45
Pentane	181	1,3-Propanediol dicaproate	221
1-Pentene	14	2,3-Propanediol dicaproate	32
Hexanal	172	1,3-Propanediol dicaproate	^a
		Dicaproin	295

Source: Le Tellier and Naivar (1972C).

^a Concentration not determined.

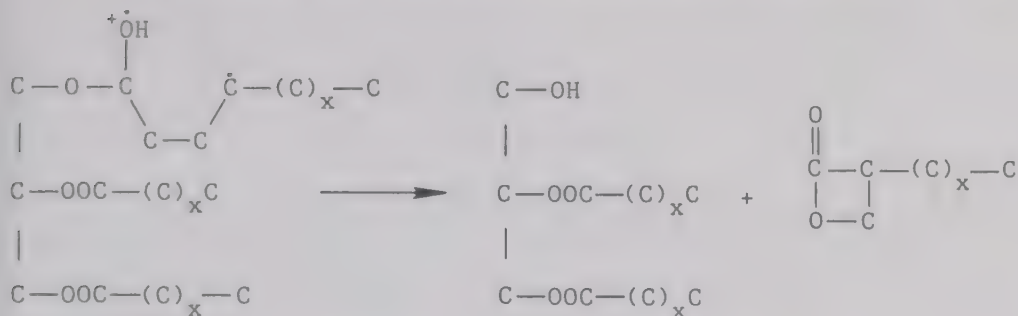
Relatively little work has been done on the radiolysis of phospholipids. We found the volatiles produced from dipalmitoylphosphatidylethanolamine (PE) to be qualitatively similar to those from palmitic acid-containing glycerides. However, the amounts of radiolytic products from the phospholipid were significantly smaller. In particular, the C_n aldehyde was partially absent. PE also produced several nonvolatile products. Among these ethanolamine phosphate and lysophosphatidylethanolamine were identified.

Mechanisms

The primary processes that occur when ionizing radiation is absorbed by matter involve the formation of excited molecules, cations, and anions. Chemical breakdown is brought about by the decomposition of these primary species and involves a number of pathways including abstraction, dissociation, radical-radical recombination, radical-radical disproportionation, and radical-molecule reactions.

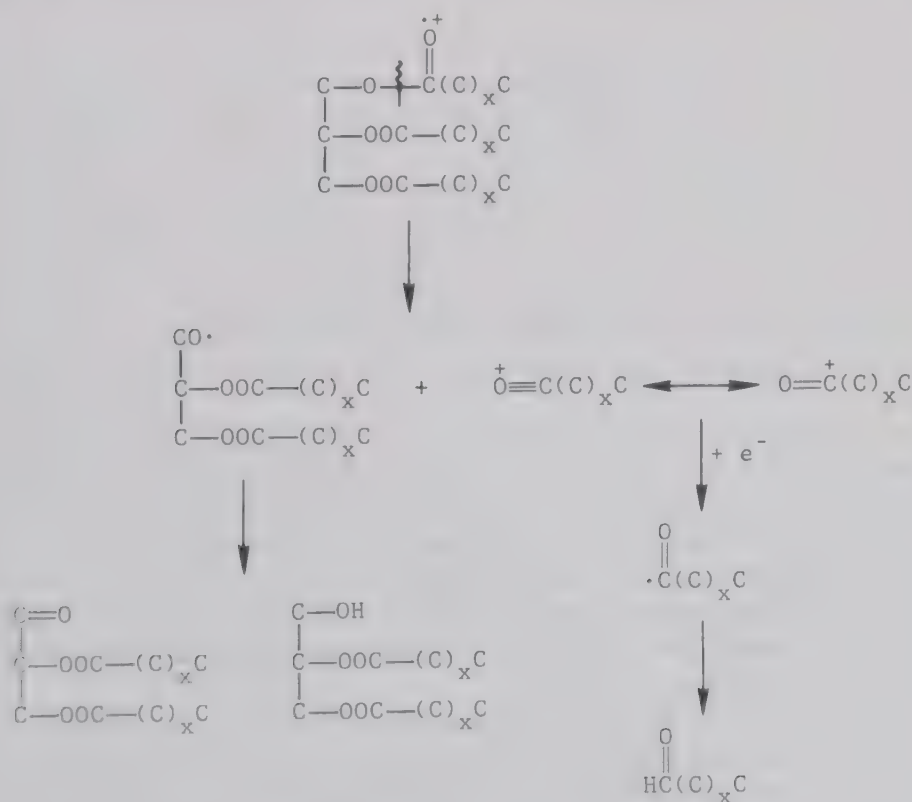
In his discussion of the elementary processes in the radiation chemistry of organic compounds, Williams (1962) suggested that in the liquid state, reactions of the parent molecule-ion involving low-activation energies probably precede the neutralization process and that threshold ionization of an organic oxygen compound involves the loss of a nonbonding electron, with the result that the unpaired electron is highly localized on the oxygen atom. The ensuing reactions were considered to be strongly directed by the tendency of the oxygen atom to complete its valence shell of electrons.

Based on the above concepts and considering the quantitative patterns obtained in our study with the triacylglycerol series, we proposed mechanisms that account for nearly all of the radiolytic compounds found. As illustrated in the simplified Schemes 1-3, the acyl radical, the propanediol diester radical, the alkyl radical, and the acyloxy radicals are seen to play an important role as major intermediates. For example, scission of the acyloxy-methylene bond in the triacylglycerol molecule-ion (Scheme 1) would produce an acyloxy free radical and a carbonium ion. The acyloxy free radical may then abstract a hydrogen atom to produce the parent fatty acid (a major radiolytic product) or decompose to give CO_2 and the C_{n-1} alkyl radical. The carbonium ion could be neutralized by electron capture (Williams 1963), forming a free radical which may abstract hydrogen from another substrate molecule to form 1,2- or 1,3-propanediol diester, depending on whether scission occurred on the primary or secondary position of the triacylglycerol. The loss of hydrogen from the propa-

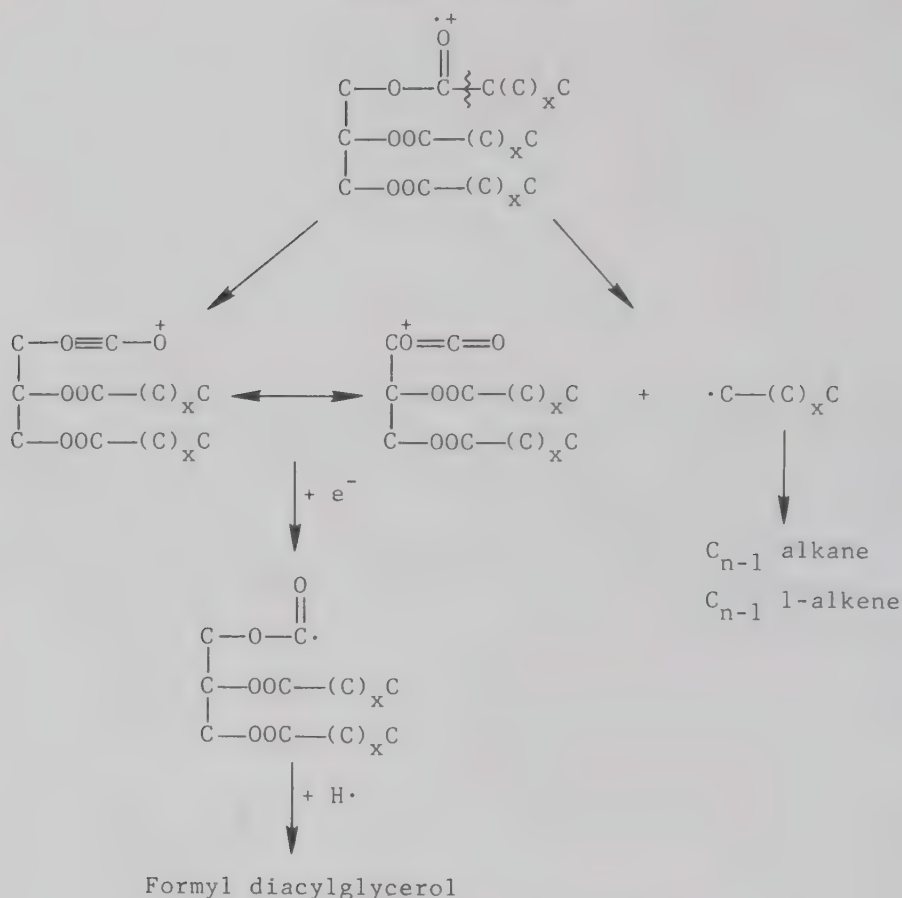


Alpha scission in the alkyl chains (Scheme 3) gives rise to the C_{n-1} alkanes and alkenes. Again, hydrogen abstraction appears to be the preferential route for termination of the alkyl radicals, since alkanes are produced in greater amounts.

As indicated previously, the C_{n-1} alkane is the most abundant of the hydrocarbon series produced from saturated fatty acids or their



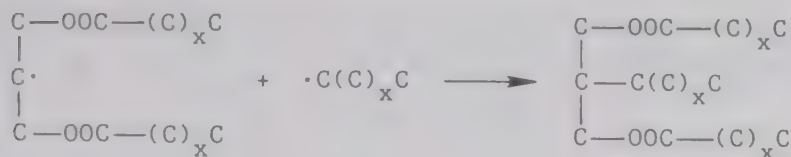
SCHEME 2. Acyl-oxy scission.



SCHEME 3. Alpha scission of alkyl chain.

esters. This is usually followed by the C_{n-2} alkene, and these two are formed in greater quantities than all other hydrocarbons. The fact that the C_{n-2} alkene is the only member of the alkene series that is often produced in more quantity than its saturated analog may be due to an intramolecular process analogous to the "McLafferty rearrangement" observed in mass spectrometry. Hydrogen transfer from the fourth carbon with a simultaneous 2,3-cleavage leads to the formation of the neutral 1-olefin.

The formation of several of the radiolytic products isolated from triacylglycerols is believed to arise from various recombinations of the above-mentioned free radicals (LeTellier and Nawar 1972C). For example, the symmetric ketone may arise by recombination of the acyl radical with the C_{n-1} radical, the alkylpropanediol diesters by recombination of the propanediol diester radical with an alkyl radical,



the glyceryl ether diesters by recombination of the propanediol diester radical with the acyl radical, and the hexanetetraol tetraester by dimerization of the propanediol diester radical.

Evidence for the general mechanisms outlined above was provided from studies in which the triacylglycerol substrate was labeled with deuterium in the glycerol moiety. Radiolytic compounds derived from alkyl, acyl, and acyloxy cleavages had no deuterium labels (Meidani *et al.* 1977).

Analysis by electron spin resonance spectrometry (ESR) provided further evidence for the radical intermediates formed by radiolysis of

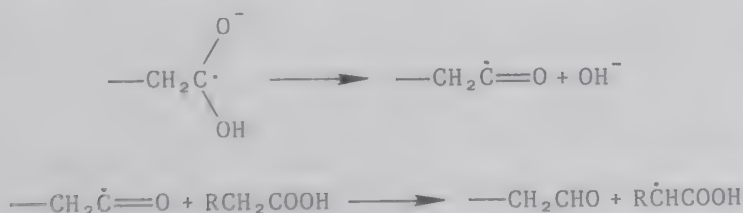
lipids. ESR spectra attributed to the anion radical $-\text{C} \cdot \begin{array}{l} \text{O}^- \\ \diagup \\ \text{OH} \end{array}$, the

α -carbon radical $\text{R}-\text{C}-\text{COOH}$, the acyl radical, the alkyl radical, the acyloxy radical, the propanediol diester radical, and (in the case of unsaturated acid substrates) the allyl radical $-\dot{\text{C}}-\text{C}=\text{C}-$, have been observed (Faucitano *et al.* 1972; Heller and McConnell 1960; Leibler *et al.* 1970; Sevilla *et al.* 1981, 1983; Taub 1983).

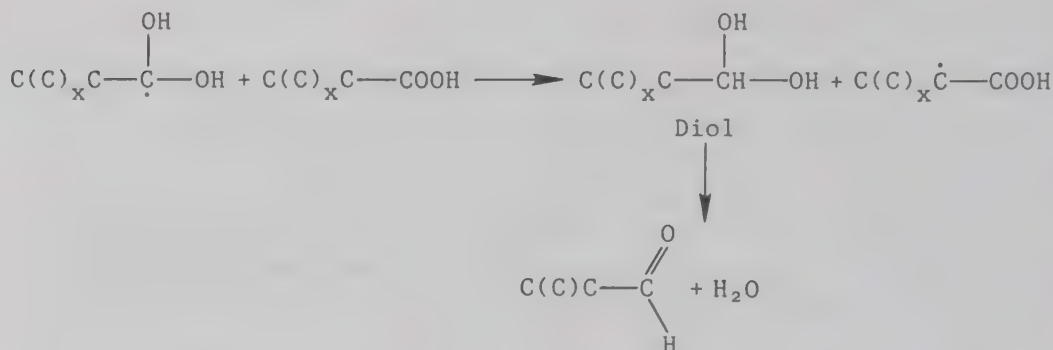
The anion radical, which arises from electron attachment to the carboxy group, has been detected following irradiation at low temperatures. At higher temperatures, it rapidly disappears and a corresponding increase in the α -carbon radical develops. The relative stability of the α -carbon radical was explained through resonance stabilization,



Faucitano and his co-workers (1972) suggested that the acyl and the α -carbon radicals arise from the radical anion as follows:



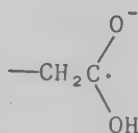
An alternative mechanism may involve direct abstraction by the protonated anion radical (Sevilla *et al.* 1981):



Faucitano's group, however, provided evidence that the reaction

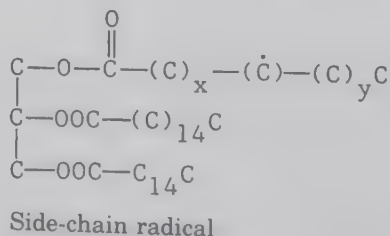
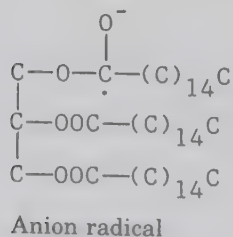


and consequently contribution of the radical anion



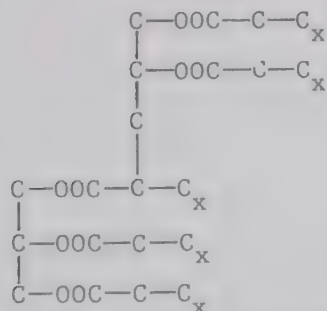
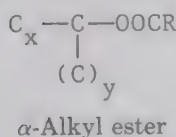
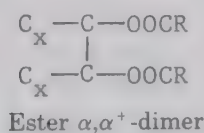
to the mechanism of radiolytic decarboxylation is of minor importance.

The recent studies of Sevilla and his co-workers (1983) provide the most detailed ESR data available to date for the radiolysis of triacylglycerols and phospholipids. In their studies with tripalmitin, γ -irradiated at 77°K, they obtained spectra corresponding to two major radicals, the anion radical and the side-chain radical:

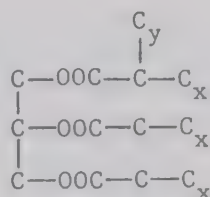


These authors suggest that the anion radical decays by abstraction of a hydrogen atom from an α -carbon atom of a parent molecule to give the α -carbon radical and a hemiacetal. The hemiacetal decomposes to yield palmitaldehyde and the diacylglycerol. Alternatively, the anion radical can decay by β -scission, producing palmitic acid and the propanediol diester radical.

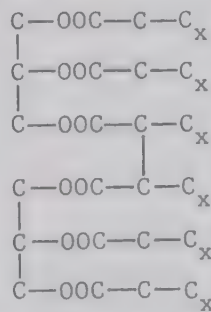
In the case of unsaturated lipids, primary ionization results in the formation of cation radicals with the charge distributed partially on the oxygen and partially on the π orbitals of the double bonds. Deportation in the region of the double bond yields the allylic radicals, which account for the formation of dehydrodimers with cross-links at positions α to the double bonds. Radical-radical combinations of the α -carbon radical account for the formation, in irradiated fatty acid esters and triacylglycerols, of adducts of the types shown below (Merritt and Vajdi 1982; Vajdi *et al.* 1983):



Propanedioldiester-triacylglycerol adduct



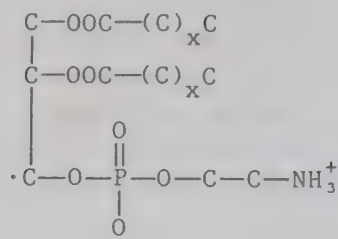
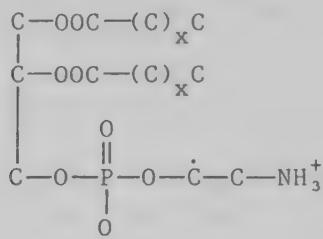
Alkyl-substituted triacylglycerol



Triacylglycerol 2,2'-dimer

In their studies with dipalmitoylphosphatidylethanolamine, Sevilla and his co-workers (1983) obtained ESR spectra which correspond to the anion, the side chain, the propanediol diester, and the α -carbon radicals observed in triacylglycerols. In addition, a radical of the phosphorylethanolamine portion of the molecule was detected. Since there are two α -carbons to the phosphate groups, two possible

structures were suggested:



THERMOLYTIC REACTIONS

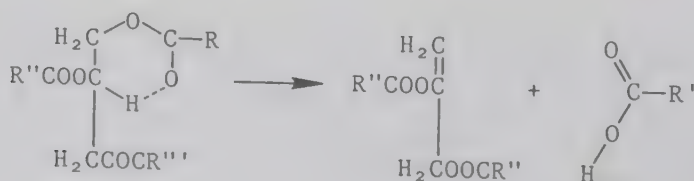
Saturated Substrates

Products. When simple saturated triacylglycerols are exposed to heat in the absence of oxygen, they give rise to predictable patterns of compounds such as hydrocarbons, free fatty acids, or symmetric ketones.

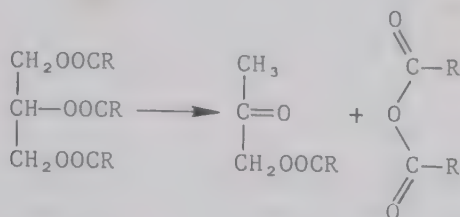
In general, very high temperatures of heating (200°–700°C) are required to produce substantial nonoxidative decomposition of saturated fatty acids. However, using sensitive techniques of analysis, thermolytic products can be detected after heating in vacuum for only 1 hr at 180°C. The pattern can be clearly seen when a homologous series of triacylglycerols are examined (Crnjar *et al.* 1981).

The specific thermolytic compounds depend on the chain length of the parent fatty acid in the triacylglycerol molecule. Typically, a simple triacylglycerol produces the following compounds (n being the number of carbons in the fatty acid moiety): a series of normal alkanes and 1-alkenes with the C_{n-1} alkane predominating; a C_n fatty acid; a C_{2n-1} symmetric ketone; a C_n oxopropyl ester; C_n propene and propanediol diesters; and C_n diacylglycerols. Acrolein, CO, and CO_2 are also formed.

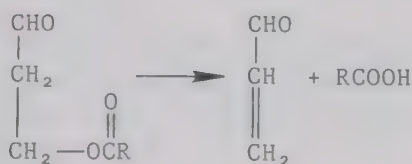
Mechanisms. In a moisture-free system, triacylglycerols may undergo a chelate type of "6-atom ring closure" by way of a hydrogen bridge. Readjustment of the electrons would give rise to the fatty acid and an olefin as follows:



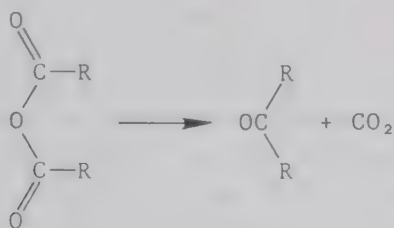
This mechanism also explains the formation of propenediol diesters. Expulsion of the acid anhydride from the triacylglycerol molecule produces 1- or 2- oxopropyl esters and the acid anhydride:



Decomposition of the 1-oxopropyl ester gives rise to acrolein and the C_n fatty acid,



while decarboxylation of the acid anhydride intermediate produces the symmetric ketone as follows:

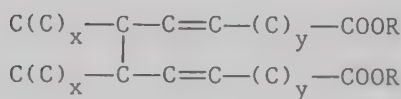


In the presence of moisture, fatty acids are released via hydrolysis of the ester linkages, a reaction requiring a molecule of water for each ester group. No evidence for positional specificity was apparent when a triacylglycerol in which the acid in the 2-position was labeled with ^{14}C was thermally hydrolyzed (Buziassy and Nawar 1968). However, Noble *et al.* (1967) noted a preferential release in favor of the unsaturated and the shorter chain acids, which was explained on the basis of their greater solubility in water.

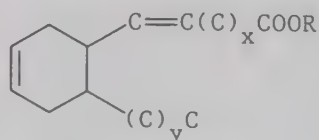
If the esterified acids possess certain functional groups such as hydroxyls, their hydrolysis by heat may result in the formation of compounds of particular flavor significance such as lactones and methyl ketones. In case of relatively high heating temperatures, the alkane and alkene series may arise by homolytic cleavage of carbon-carbon bonds along the fatty acid chains.

Unsaturated Substrates

Products. Dimeric, polymeric, and cyclic compounds appear to be the predominant thermolytic products of unsaturated fatty acids. The dimers include alicyclic mono-, di-, and triene dehydrodimers, saturated dimers with cyclopentane structures, tetrasubstituted cyclohexenes, and bicyclic and tricyclic dimers (Figge 1971; Sen Gupta 1966; Sen Gupta and Scharmann 1967):



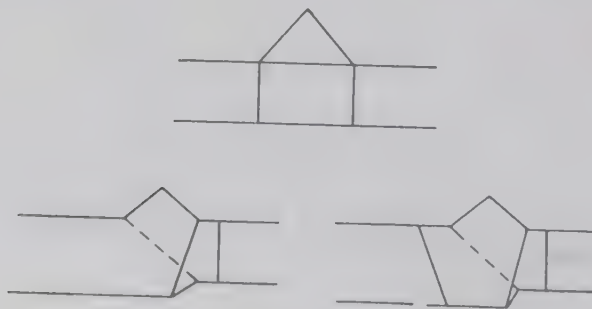
Dehydrodimer



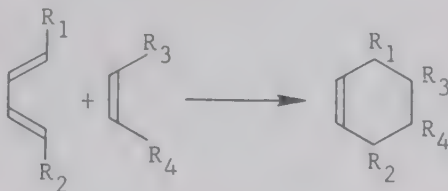
Cyclic monomer

Cyclic monomers have been shown to be toxic to animals, hence the level at which they are generated in fats exposed to high temperatures, as in the case of frying, is of major concern. Intensive research on this subject is presently under way in several laboratories.

Mechanisms. The dimeric compounds arise via combination of allyl radicals that result from hydrogen abstraction at methylene groups α to double bonds. Such radicals may undergo disproportionation, or inter- and intramolecular addition to C—C double bonds. The latter mechanism is thought to be responsible for the formation of a variety of cyclic structures:



Dimerization of unsaturated fatty acids can also occur via Diels–Alder reactions:



In the case of triacylglycerols, dimerization can take place between acyl groups in two triacylglycerol molecules or between two acyl groups in the same molecule. If sufficient double bonds are available, as in dimers of polyunsaturated fatty acids, further reaction may take place, producing trimers and polymers. Obviously, the larger the number of double bonds in the fatty acid chain and the higher the temperature of heating, the greater are the chances for thermal dimerization, polymerization, and cyclization.

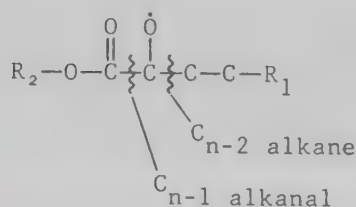
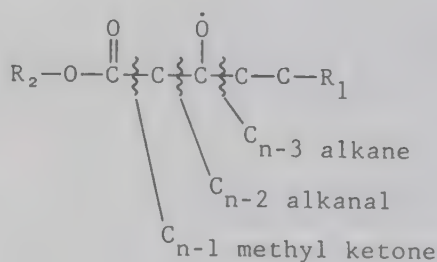
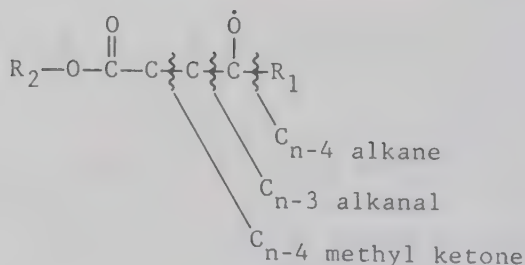
It should be pointed out that both thermal dimers and cyclic monomers are also produced under oxidative conditions. In the presence of oxygen, however, they are formed more rapidly due to the role of peroxy radicals in hydrogen abstraction.

THERMAL OXIDATIVE REACTIONS

Saturated Substrates

Products. Saturated fatty acids, their esters, and their triacylglycerols are relatively stable. However, when heated in air at temperatures higher than 150°C , they produce a variety of oxidation products which can be detected by simple techniques (Crnjar *et al.* 1981). Each triacylglycerol gives rise to a homologous series of hydrocarbons, aldehydes, ketones, and lactones. The hydrocarbons are the same as those produced in the absence of oxygen, i.e., homologous series of n -alkanes and 1-alkenes, with the alkanes predominating and the decarboxylation product of the parent fatty acid produced in substantial amounts. However, the amounts formed are much greater under the oxidative conditions. The methyl ketones are generally produced in larger quantities than the alkanals, with the C_{n-1} methyl ketone being the major carbonyl compound formed. In each lactone series, the C_n lactone is the most abundant. However, the only δ -lactones formed are those having a carbon number equal to that of the parent fatty acid. The amounts of the $C_n\gamma$ - and δ -lactones decrease with increased fatty acid chain length.

Mechanisms. It is generally accepted that the principal mechanism in thermal oxidation of saturated fatty acids involves the formation of monohydroperoxides and that oxygen can attack all the methylene groups of the fatty acid chain. The observation, made by many investigators, that the dominant oxidative products of saturated fatty acids are those with chain lengths near or equal to the parent fatty acids led to the conclusion that oxidation occurs preferentially at the α , β , and γ positions. As shown below, subsequent cleavage in the region of the alkoxy radical leads to a favored formation of the longer chain hydrocarbons, alkanals, and methyl ketones.

 α -Attack β -Attack γ -Attack

Unsaturated Substrates

Products. In general, the decomposition products found in thermally oxidized lipids are qualitatively similar to those identified in the same lipids when autoxidized at low temperature. These include series of aldehydes, ketones, acids, esters, alcohols, hydrocarbons, lactones, cyclic compounds dimers, and polymers. The quantitative patterns of the decomposition products formed at elevated temperatures, however, are quite different from those of autoxidation. Such differences vary widely depending on the nature of the substrates and the parameters of the heat treatment.

Mechanisms. Unsaturated fatty acids are much more susceptible to oxidation than their saturated analogs. Their autoxidation has been extensively reviewed in the literature and is discussed in other chapters in this volume. Although specific differences between high- and low-temperature oxidations have been observed, the evidence accumulated to date indicates that in both cases the principal reaction pathways are essentially the same, i.e., via the formation and decomposition of hydroperoxide intermediates. In some cases the relative proportions of the isomeric hydroperoxides isolated from each substrate varied with oxidation temperatures in the range 25°–80°C. However, their qualitative pattern was the same (Frankel 1979). At oxidation temperatures higher than 80°C, isolation or quantitation of hydroperoxide intermediates is difficult, if not impossible, because of their extreme sensitivity. When ethyl linolenate, for example, was heated in air at 250°C, hydroperoxide decomposition occurred so rapidly that a net peroxide value of zero was reached in less than 30 min (Lomanno and Nawar 1982).

Not only is the formation and destruction of hydroperoxides extremely rapid at high temperatures, but also the resulting primary decomposition products are themselves unstable and rapidly undergo further oxidative decomposition. As the oxidative process continues, a variety of possible reaction mechanisms come into play, leading to the formation of relatively complex decomposition patterns. It is not surprising, therefore, that although the primary oxidative events appear to be the same over a wide temperature range, correlation between the pattern of end products and classic carbon–carbon cleavage of the expected hydroperoxide intermediates becomes more and more evasive as the temperature of oxidation or the degree of unsaturation (of fatty acid substrate) is increased. Thus, less of the “expected” products have been accounted for in linolenates than in linoleates and less in linoleates than in oleates after oxidation at high temperatures. Furthermore, a number of “unexpected” compounds varying widely in both structure and concentration have been found.

Indeed, the number of compounds that have been identified in thermally oxidized lipids is literally in the hundreds (Chang and Paulose 1973; Chang *et al.* 1978; Frankel 1982; Selke *et al.* 1975, 1980; Nawar *et al.* 1977).

In our work with ethyl linoleate heated at temperatures from 70° to 250°C, 11 predominant compounds were observed in major quantities and, in addition, numerous other compounds were found in relatively small amounts. Typical of the expected products are pentane and hexanal from decomposition of the 13-hydroperoxide, and 2,4-decadienal, ethyl octanoate, and ethyl-9-oxononanoate from decomposi-

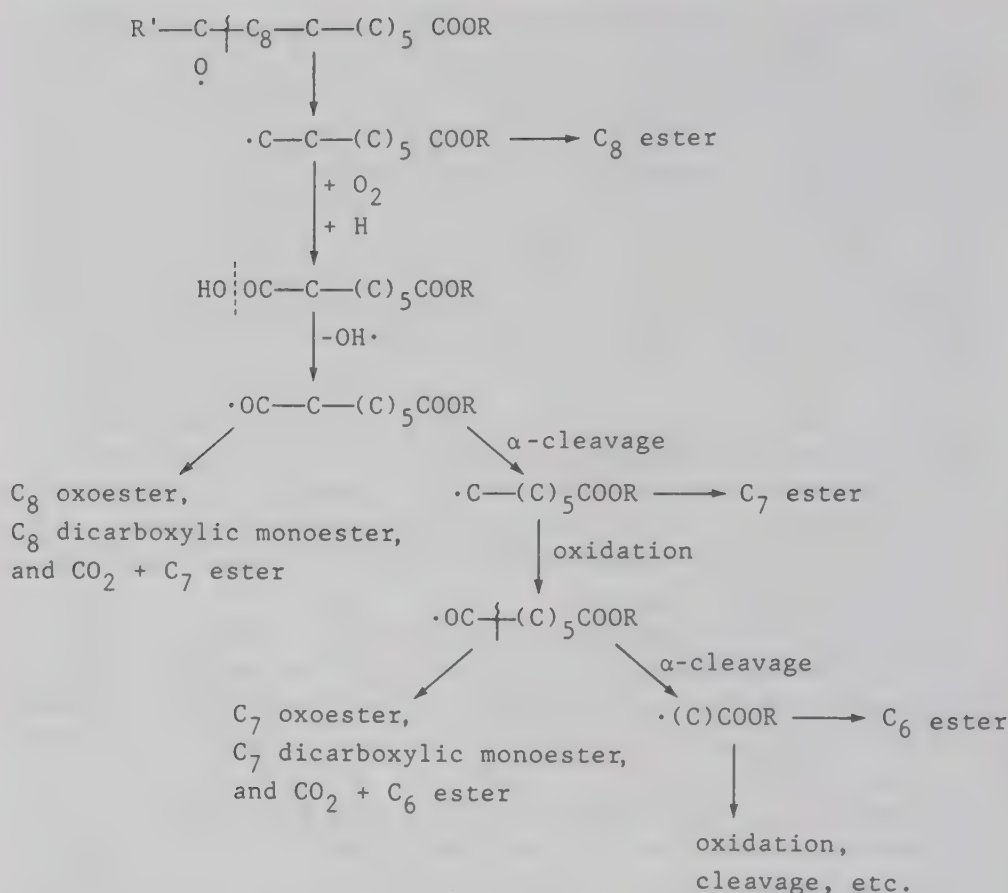


FIG. 6.1. Possible mechanism for the formation of the short-chain esters, oxoesters, and dicarboxylic acids.

tion of the 9-hydroperoxide isomer. The C₈ aldehyde ester, a compound not predictable from any of the "confirmed" hydroperoxides of linoleate but found in relatively large amounts, may be produced by cleavage of the 9-alkoxy radical followed by terminal hydropreoxidation, as shown in Fig. 6.1. Further oxidation of the C₈ aldehyde ester would produce the corresponding acid HOOC(CH₂)₆COOC₂H₅ which, upon decarboxylation, would give rise to ethyl heptanoate. Both the C₈ dicarboxylic acid and the C₇ ethyl ester are major products of linoleate thermal oxidation. The 8-alkoxy radical may also decompose to give formaldehyde and the C₇ alkyl radical, which would produce ethyl heptanoate or form a terminal hyperperoxide, and so on. This mechanism explains the formation of C₈, C₇, C₆ aldehyde ester and dicarboxylic acid series, as well as the C₇, C₆, C₅ ethyl ester series which are found in decreasing concentrations as their chain length shortens.

In general, these compounds are formed in only trace amounts in case of room-temperature oxidation.

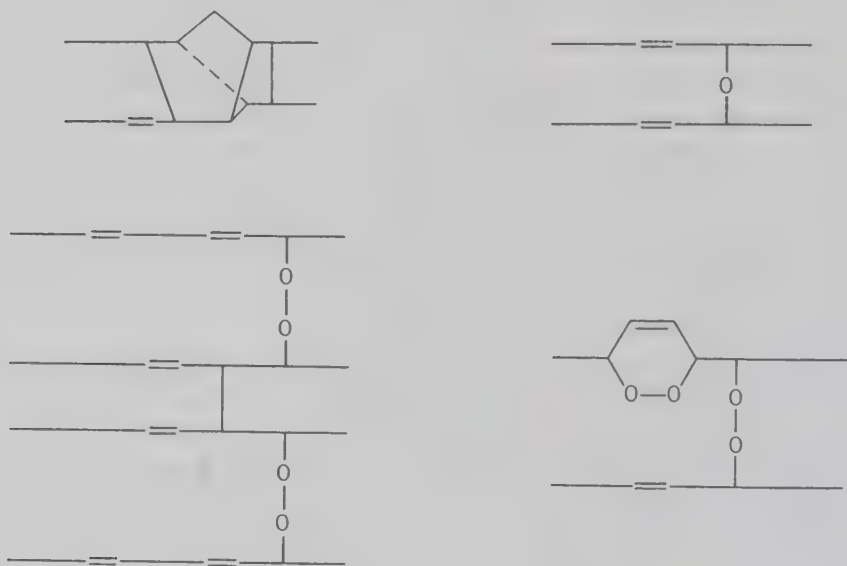
An alternative mechanism for the formation of the C₈ aldehyde ester and the C₇ ethyl ester is via formation and decomposition of the unconjugated C₈ hydroperoxide. Oxygen attack outside the 1,4-pentadiene system of linoleate is considered unlikely; the C₈ or C₁₄ linoleate hydroperoxides have never been isolated. However, all the available information regarding the identity and relative distribution of hydroperoxide intermediates was obtained from oxidations at 80°C or lower. It is conceivable that formation of the C₈ or C₁₄ hydroperoxides may be enhanced at the higher temperatures. Indeed, many authors speculated that hydrogen abstraction at allylic positions outside pentadiene systems is responsible for many of the "unpredicted" decomposition products found among the high-temperature decomposition products. Such mechanisms include those proposed by Michael (1966) to rationalize the formation of aromatic compounds and cyclic monomers from methyl linoleate, and by Noble and Nawar (1975) to explain the production of 2-cyclohexenyl acetate and 5-cyclohexenyl pentenoate from methyl docosahexaenoate.

Quantitative comparison of some of the major compounds produced from propyl linoleate at different heating temperatures shows very little difference between 180° and 250°C. However, hexanal and propyl-9-oxononanoate were found in significantly greater amounts in the samples heated at 70°C than those heated at the higher temperatures. The reverse was true for *t,t*-2,4-decadienal. These results are in agreement with the observations made by Kimoto and Gaddis (1969) and Swoboda and Lea (1965), who reported that hexanal was the major volatile compound formed during low-temperature autoxidation, while 2,4-decadienal predominated on thermal decomposition. It was speculated that the so-called "type B" scission of the alkoxy radical (cleavage of the C—C bond between a vinyl function and the oxygen-bearing carbon atom) is favored at low temperatures, while "type A" (cleavage splitting of the C—C bond on the side away from the olefinic linkage) occurs preferentially at elevated temperatures. On the other hand, decadienal was found in considerably greater amounts in our samples of vegetable oils when heated at 185°C for 2 hr than in the oils heated at 250°C, while the amounts of hexanal were approximately the same for both treatments.

Some of the hydroperoxides typical of linolenate, and even certain products of hydroperoxide cleavage, contain 1,4-pentadiene systems and would thus continue to undergo further hydroperoxide formation followed by scission at extremely rapid rates. Also unique to the oxidation of linolenates is their tendency to form polyhydroperoxides,

hydroperoxy epoxides, and hydroperoxy cyclic peroxides. The formation and decomposition of such secondary oxidation products have been discussed in detail by Frankel (1982).

Radical combination of the alkoxy or the peroxy radicals, formed in unsaturated lipids or their intra- or intermolecular addition to carbon-carbon double bonds, lead to the formation of oxydimers or polymers possessing hydroperoxide, hydroxide, epoxide, carbonyl, or cyclic groups as well as ether and peroxide bridges. Some examples follow:



The formation of polycyclic aromatic hydrocarbons via pyrolysis of the lipids at temperatures exceeding 400°C has been of some concern due to their carcinogenicity (Lijinsky and Shubik 1964; Halaby and Fagerson 1971). Although such temperatures are not common in food processing, the formation of these compounds is possible if certain portions of the food or its fat drippings come in contact with charcoal or very hot surfaces, as may occur in broiling or roasting. The general mechanism proposed by Badger and Novotny (1963), based on repeated condensation and cyclization of 2-carbon intermediates (Fig. 6.2), may account for the production of these aromatic hydrocarbons not only from the food lipids, but from the nonlipid nutrients as well.

COMPARISON OF PRODUCT PATTERNS

It is clear from the above discussion that the mechanisms involved in thermolytic, oxidative, and radiolytic reactions are quite different.

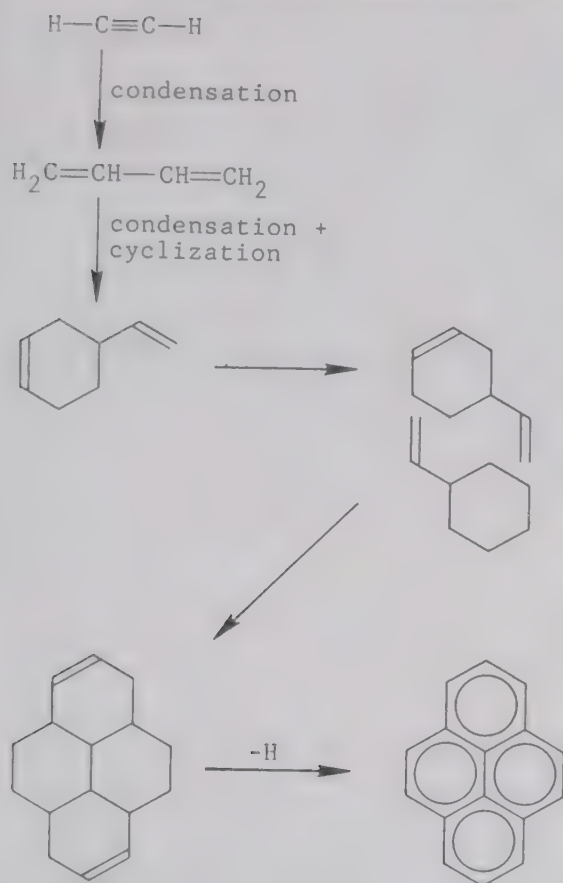


FIG. 6.2. Mechanism for the formation of polycyclic aromatic hydrocarbons (according to Badger and Novotny 1963).

It is equally clear that many of the typical products of decomposition are the same for all three processes. The total decomposition patterns, however, are sufficiently different that the typical effect of each individual process remains recognizable even if the treatments are superimposed. Selected examples from a recent comparative study of the volatile patterns of heated and irradiated model systems (Witchwoot and Nawar 1982) are given here.

The polar volatile compounds produced from ethyl stearate by heat (180°C , 1 hr in air), by irradiation (250 kGy), and by irradiation and heat are shown in Fig. 6.3. Seen in the top chromatogram are the series of aldehydes, ketones, and esters expected from thermal oxidation of saturated systems. Irradiation produced the homologous series of ethyl esters (arising from carbon—carbon cleavages along the alkyl chain), but not the shorter chain aldehydes or ketones (middle chromatogram). When both radiation and heat treatments were applied, the aldehydes and ketones were more prevalent.

For the nonpolar fraction (Fig. 6.4), heat and irradiation produced the same homologous series of hydrocarbons, but the relative amounts

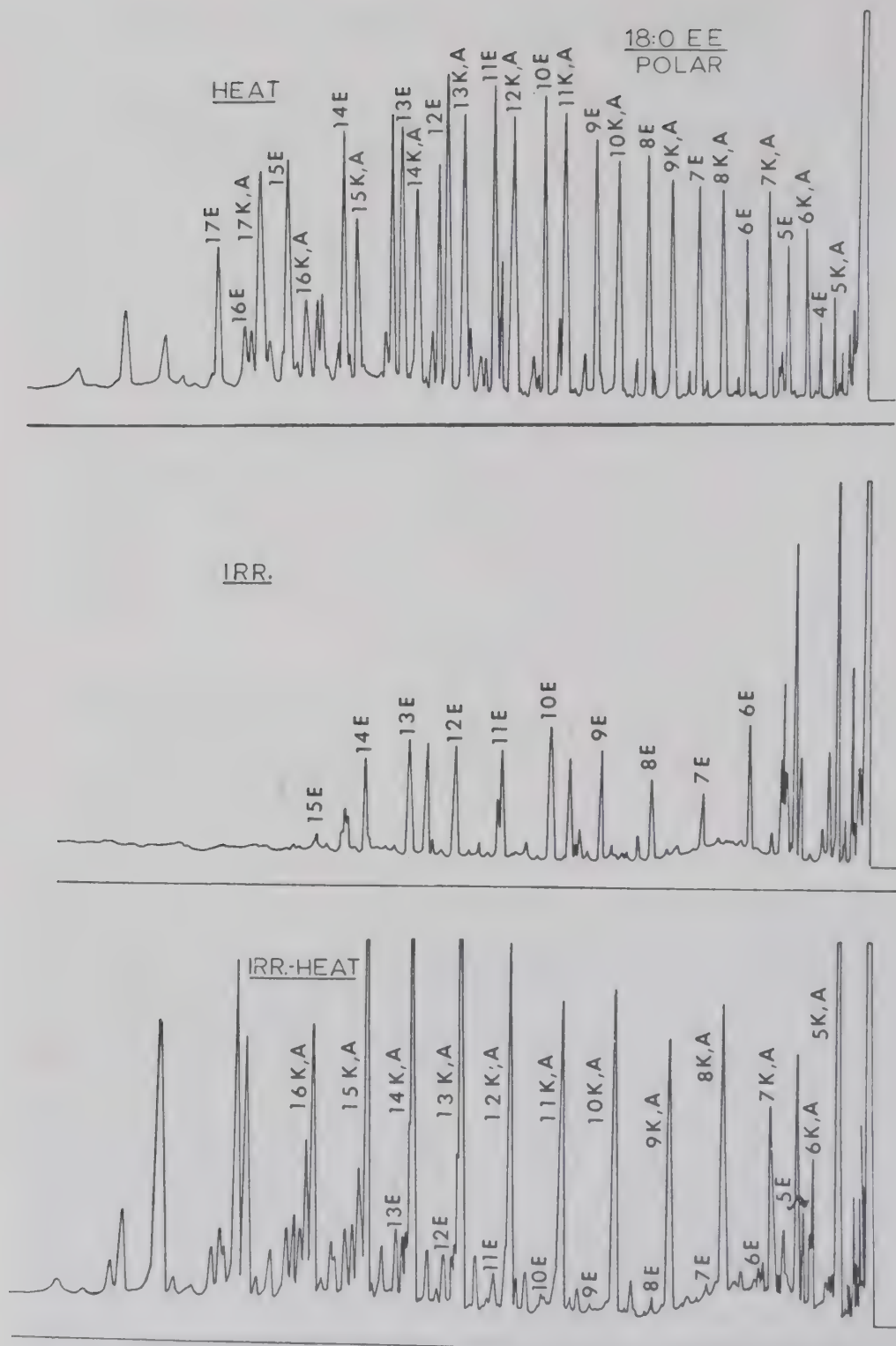


FIG. 6.3. Comparison of the polar volatile compounds produced from ethyl stearate by heat (180°C, 1 hr) and irradiation (250 kGy). Numbers indicate carbon chain length. K, methyl ketone; A, alkanal; E, ethyl ester.

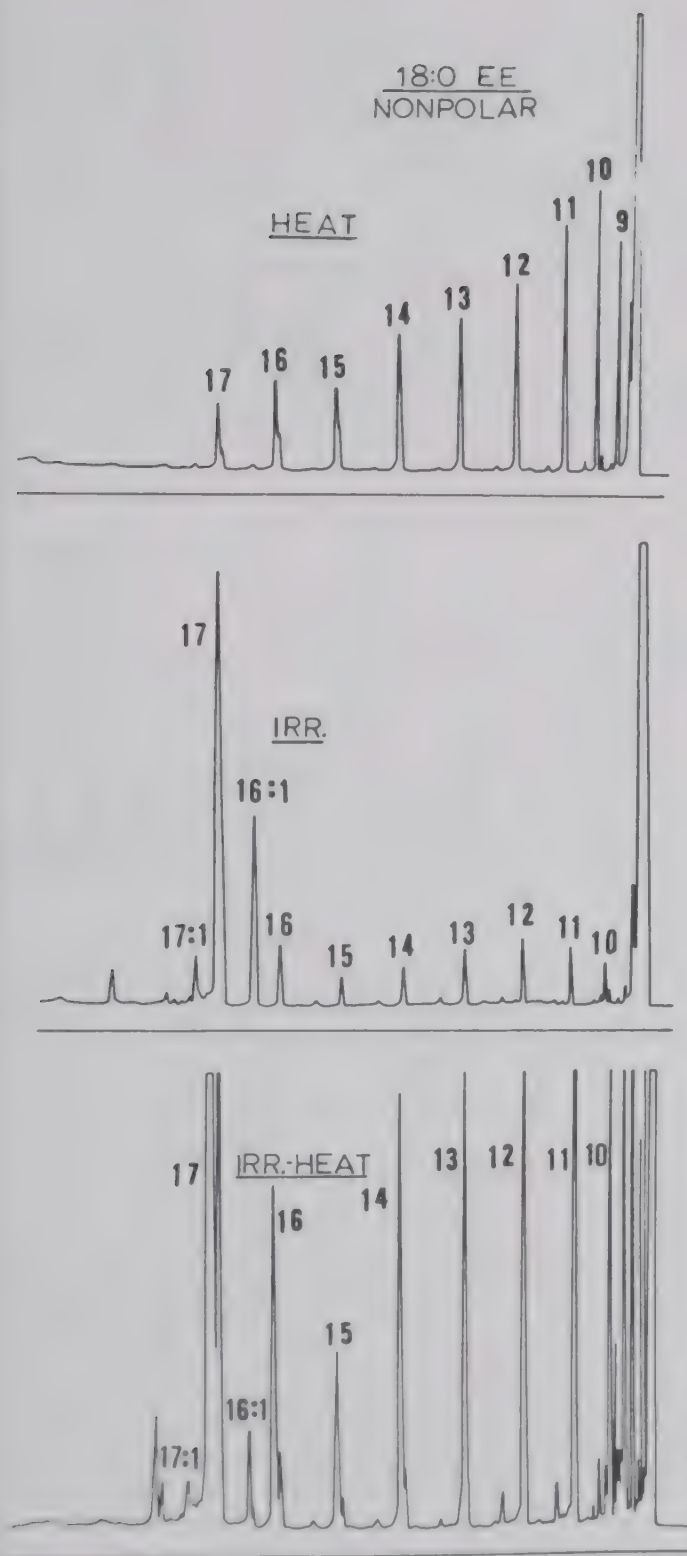


FIG. 6.4. Comparison of the nonpolar volatile compounds produced from ethyl stearate by heat (180°C, 1 hr) and irradiation (250 kGy). Numbers indicate carbon chain length of *n*-hydrocarbons.

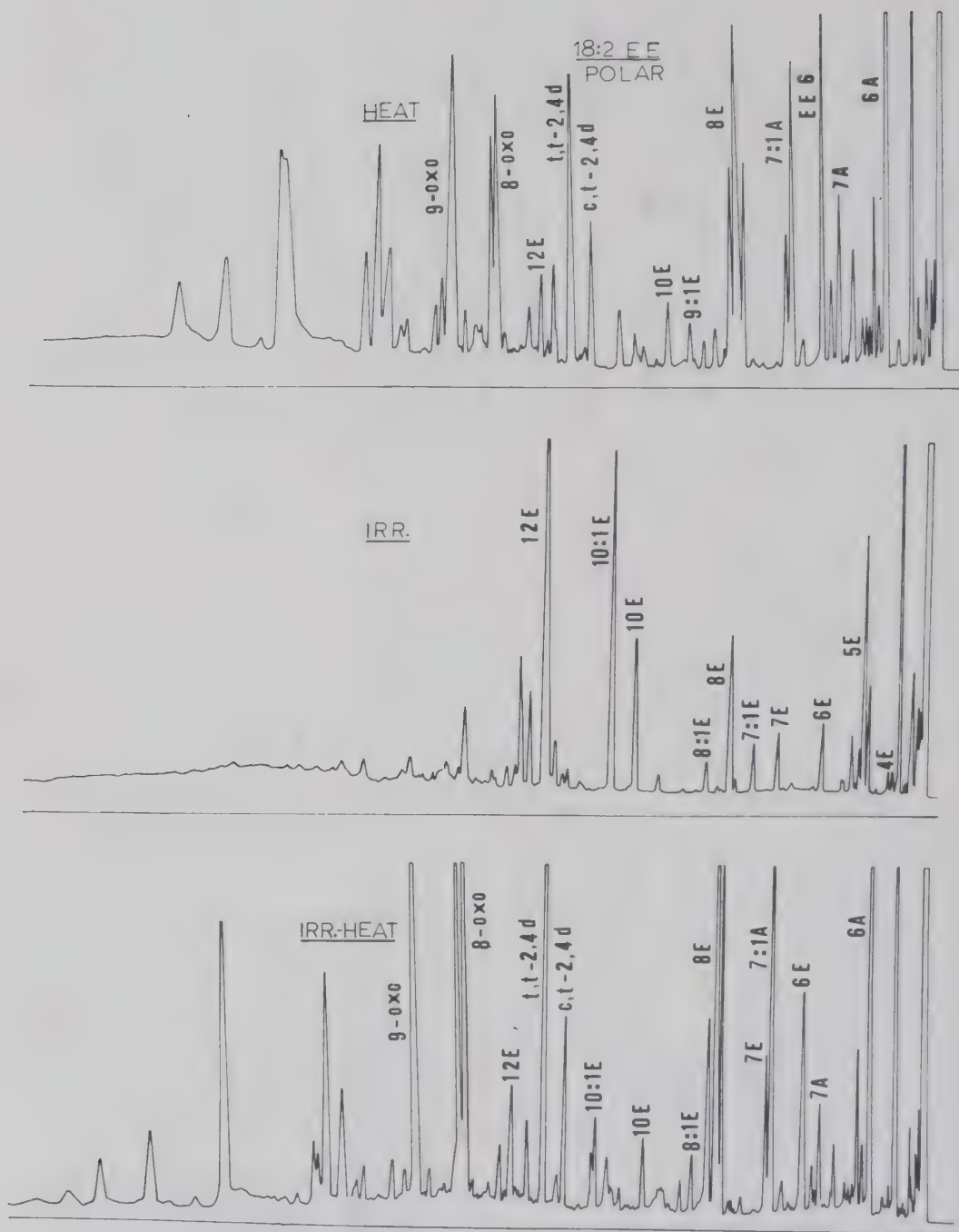


FIG. 6.5. Comparison of the polar volatile compounds produced from ethyl linoleate by heat (180°C, 1 hr.) and irradiation (250 kGy). Numbers indicate carbon chain length. A, aldehyde; E, ethyl ester; 2,4d, 2,4-decadienal; oxo, aldehyde ester.

were different. In the case of irradiation, the alkane of one carbon atom less than the parent fatty acid is by far the major hydrocarbon, and the alkene of two carbons less than the substrate is the most abundant of the unsaturated series.

An example of an unsaturated substrate, ethyl linoleate, is given in Fig. 6.5. Heating produced the typical pattern of linoleate oxidation, hexanal, 1,4-decadienal, ethyl octanoate, and the 9- and 8-oxoesters, while irradiation produces the homologous series of ethyl esters. When both treatments are applied, the oxidation products dominated the volatile decomposition pattern.

More detail regarding the volatile patterns from several substrates will be published elsewhere. We are now conducting a similar comparison of the more complex dimeric and polymeric products.

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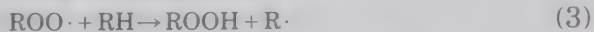
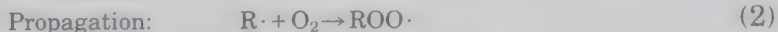
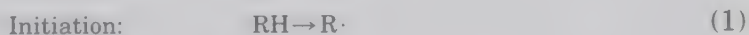
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Antioxidants

Michael G. Simic and Edward P.L. Hunter¹

INTRODUCTION

Autoxidation is a peroxidation process induced by atmospheric oxygen. It is a free radical chain process propagated by peroxy radicals which can greatly amplify the effects. Autoxidation is responsible for the deterioration of various organic materials ranging from the biologically important (e.g., lipids, foods, cell membranes) to the industrially important ones (e.g., rubber, lubricants). The mechanism of autoxidation of an organic substrate, RH, is very well understood and is described by reactions 1–4 (Ingold 1961, 1969, 1971; Mahoney 1969; Howard 1973).



Formation of the hydroperoxides, ROOH, and their decomposition products are actually responsible for the rancidity of fats and oils, the brittle properties of deteriorating rubber, etc. It has been known for some time that phenols, HArOH (where HAr stands for an aromatic group), and aromatic amines are effective antioxidants that inhibit

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the autoxidative chain by intercepting $\text{ROO}\cdot$ by reactions 5 and 6, thereby diminishing the autoxidation chain length.



Reaction 6 has not been directly demonstrated and is less important because of the low steady-state concentrations of reactants in naturally oxidizing systems. The rate-determining step in this mechanism is reaction 3. For the uninhibited case and for a given chain initiation rate, v_i ; the radicals $\text{R}\cdot$ and $\text{ROO}\cdot$ attain a steady-state concentration and the rate of oxygen uptake is given by I:

$$-d[\text{O}_2]/dt = k_3(v_i/2k_4)^{1/2}[\text{RH}] \quad (\text{I})$$

With regard to lipid peroxidation, the most widely studied antioxidants have been phenols. These include the naturally occurring tocopherols that comprise vitamin E and the man-made and widely used food preservatives such as BHT and BHA. For a system containing such an antioxidant as chain inhibitor, reactions 5 and 6 replace reaction 4 as the important peroxy radical removing process so that O_2 uptake is governed by Eq. II:

$$-d[\text{O}_2]/dt = v_i k_3 [\text{RH}] / 2k_5 [\text{AH}] \quad (\text{II})$$

EXPERIMENTAL TECHNIQUES

Gas Absorption Technique

Equations I and II reveal how the course of autoxidation and its inhibition by phenolic antioxidants can be monitored by measuring O_2 uptake using the gas absorption technique (French *et al.* 1935; Howard and Ingold 1969). In this method, the organic substrate to be oxidized is contained in a reaction vessel that is connected to a gas pressure transducer, but is otherwise completely closed and gas tight. The substrate is either in the form of a neat liquid or dissolved in an inert liquid solvent or an actual food sample. Above the liquid phase is oxygen gas at pressures generally ranging from 100 to 760 Torr and which is dissolved in the liquid to an equilibrium concentration. The reaction vessel is carefully thermostated and the O_2 pressure above the liquid is automatically and continuously monitored and displayed by a suitable recording device. When air is used, the decrease of O_2 is measured by gas chromatography at suitable time intervals. The rate of consumption of O_2 is then related to either Eq. I or II. Autox-

idation is usually accelerated by varying the temperature to about 60°–70°C. It is important that the liquid phase always be saturated with O₂, which may be difficult if the substrate is oxidizing too rapidly. Minimizing the ratio of the liquid phase volume to the surface area exposed to O₂ ensures that such saturation is maintained.

Much of the earlier work in this field involved measuring the amount of peroxides formed as well as O₂ uptake in samples of lard or other lipid systems (Olcott and Emerson 1937; Golumbic 1941; Hove and Hove 1944; Griewahn and Daubert 1948; Kunkel 1951; Lea and Ward 1959; Lea 1960; Parkhurst *et al.* 1968; Olcott and Van der Veen 1968; Kanno *et al.* 1970A, B) in which the autoxidation is self-initiated. The induction periods, i.e., the times during which the oxidation is inhibited and governed by Eq. II, were used to determine the comparative effectiveness of the added antioxidants in terminating the chain by reaction 5. More recently, Burton and Ingold (1981) have pointed out the pitfalls encountered in measuring relative rate constants in which the chain initiators or the chain initiation rate are not accurately known, quantities that are required to use either Eq. I or II. To have an accurate v_i , they studied oxidizing systems containing well-characterized radical generators (e.g., from the thermal decomposition of 1,1'-azobutane and its derivatives) whose radical production rate is known and which is much greater than that due to any spontaneous reactions.

Pulse Radiolysis–Kinetic Spectrophotometric Method

In the gas absorption technique, it is necessary to know k_3 as well as v_i , but other methods have been applied to the study of reactions of antioxidants that make it possible to focus specifically on reaction 5 without the need to know any other rate constants. One such method was the pulse radiolysis–kinetic spectrophotometric (PRKS) technique which has been described in detail (Hart and Anbar 1970; Swallow 1972). Typically, a quartz cell containing a liquid sample is subjected to a brief pulse (5 nsec–1 μ sec) of ionizing radiation, usually high-energy electrons from machine sources (e.g., Linacs, Van de Graaff generators) accelerated to 2 MeV or greater energies. Such pulses produce homogeneous amounts (10^{-6} – 10^{-5} M^2) of free radicals via ionization and excitation processes. An analyzing light beam, passed through the cell and at a right angle to the ionizing pulse beam, is used to monitor the levels of these free radicals and the products resulting from their reactions. The transmitted light is dispersed by a

² M stands for molar dm⁻³.

monochromator, and the time dependence of the intensity at a particular wavelength is monitored by fast photodetectors (e.g., photomultipliers, photodiodes) whose output signals are recorded with a transient analyzer. By converting the transmission signals into absorbance (the quantity directly proportional to free radical concentrations) and subjecting the data to kinetic analysis, one obtains desired rate constants. In the work described here, peroxy radicals were generated in systems containing a large excess of antioxidants so that their reactions were pseudo-first order. Plots of pseudo-first order rate constants vs antioxidant concentrations (0.2–50 mM) gave linear dependencies whose slopes yielded bimolecular rate constants for reaction 5. The success of this method depended on the reactant and product radicals having sufficiently different spectra with large enough extinction coefficients ($> 500 \text{ M}^{-1} \text{ cm}^{-1}$) in the visible or ultraviolet (UV) region.

The generation, reactions, reactivities, and lifetimes of peroxy and aroxy radicals are discussed in succeeding sections.

Flash Photolysis–Electron Spin Resonance

Since all of the elementary steps involved in the autoxidation process involve odd-electron species, a method uniquely suited to monitoring the reactions would employ electron spin resonance (ESR) spectroscopy (Ayscough 1967; Ingram 1969). Such a method is flash photolysis–electron spin resonance (FPESR) which combines techniques that generate, detect, and identify free radicals and thus shares some characteristics with the PRKS method (Bolton and Warden 1974). The FPESR technique uses a brief pulse of UV light to photochemically generate free radicals in an ESR cell within the cavity of an ESR spectrometer. Techniques exist to measure the ESR spectrum of transients by rapidly scanning the spectrometer's magnetic field. Then the time dependence of radical reactants and products can be followed by tuning the spectrometer to a particular ESR peak and time resolving the signal with a transient analyzer.

A key advantage of using ESR to detect radical transients is the wealth of structural information that can usually be obtained which permits more confident identification of the radical than can generally be obtained from optical spectra. Also, many radicals can be detected by ESR that cannot be easily detected optically because they absorb at quite short wavelengths, as is the case with many alkyl radicals. The optical and ESR spectral properties of peroxy and aroxy radicals are discussed in a later section.

GENERATION OF PEROXY RADICALS

Peroxy radicals were generated in polar and nonpolar solvents. Since many antioxidants are sparingly soluble or insoluble in aqueous solutions, the majority of experiments were conducted in nonpolar solvents. Some of the hydroxy-aromatic (hydroxy aryl, HArOH) antioxidants are somewhat soluble in the HArO^- form in alkaline solutions, i.e., at $\text{pH} > \text{pK}_a \sim 10.5$.

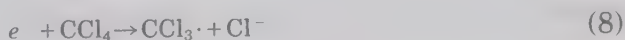
Nonpolar Solvents

Radiolysis of cyclohexane, $c\text{-C}_6\text{H}_{12}$, generates cyclo-hexyl radicals which in the presence of oxygen give cyclo-hexyl peroxy radicals (Simic and Hayon 1971).

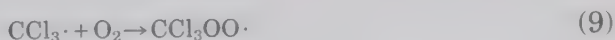


In air-saturated solutions the formation of peroxy radicals is achieved in less than $1 \mu\text{sec}$.

In chlorinated solvents the radiation-generated electrons are utilized to produce radicals, e.g. (Buhler and Hurni 1978; Brede *et al.* 1982),

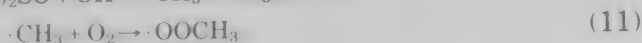


followed by



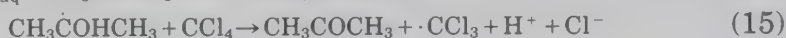
Aqueous Solutions

The radiolysis of water produces e_{aq}^- , OH, and H atoms as primary radicals with respective G values (number of species formed from 100 eV energy absorbed) of 2.8, 2.8, and 0.55. These species are utilized for the generation of a variety of organic radicals and their corresponding peroxy radicals. For example,



Carbon tetrachloride is sparingly soluble in water. Its solubility can be increased by addition of alcohols and ketones which also act to

convert the primary water radicals into species capable of reducing CCl_4 . Thus, the radiolysis of CCl_4/i -propanol/acetone aqueous solutions produces $\text{CCl}_3\cdot$ as follows (Packer *et al.* 1980):



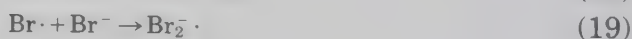
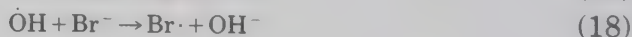
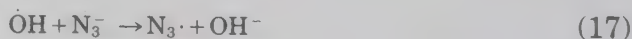
These reactions are then followed by reaction 9.

GENERATION OF OXIDIZING RADICALS

In aqueous solutions a variety of oxidizing radicals can be generated. For example (Simic and Hunter 1984A, B),



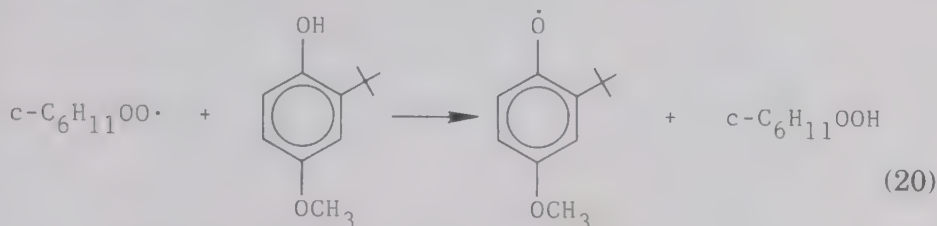
The carbonate radical is a strong oxidizing agent, but fairly unreactive with CH bonds and unsaturated groups (Chen and Hoffman 1973). Hence, it is an ideal agent for generating aroxy radicals. Other frequently used oxidizing radicals are azide ($\text{N}_3\cdot$) and bromide ($\text{Br}_2\cdot$) radicals generated by OH.



REACTIONS OF PEROXY AND OXIDIZING RADICALS WITH ANTIOXIDANTS

As we have pointed out, these reactions can be conducted in polar (aqueous) and nonpolar media. This choice of testing antioxidants in polar and nonpolar media is rather important not only from the point of view of solubility of antioxidants, but also because autooxidative processes in natural products can take place in both membranes/fats as well as in inter- and intracellular fluids. It could be argued that autooxidation in membranes/fats due to long-chain reactions is more important from the quantitative point of view. However, autooxidation in inter- and intracellular fluids could also be important from the qualitative point of view. Antioxidants can be defined as good H atom donors whose resulting radicals do not propagate autooxidation. For

example, BHA readily reacts with both $c\text{-C}_6\text{H}_{11}\text{OO}\cdot$ and $\text{CCl}_3\text{OO}\cdot$ radicals. The reactive group in this type of antioxidants is the hydroxy group (Simic 1981),



The BHA radical is usually represented with a general form $\text{HArO}\cdot$ where $\text{—O}\cdot$ indicates predominant localization of the unpaired electron on the oxygen of the hydroxy group. For phenolic derivatives these $\text{HArO}\cdot$ radicals are designated as phenoxy radicals or in general aroxy radicals (aryl-oxy).

The above reaction will take place both under steady-state and pulsed conditions. If the peroxy radicals are generated continuously, a steady-state concentration of peroxy radicals and antioxidant radicals will be achieved. The steady-state concentrations will depend on the rate of generation of peroxy radicals, peroxy radical reactivity, antioxidant concentration, and antioxidant reactivity. This steady-state condition can be achieved by cobalt-60 γ -ray irradiation and is representative of natural autooxidation processes which can take place from minutes to months. This type of process is not amenable to kinetic measurements and the study of intermediates.

If the peroxy radicals are generated in a short period of time, e.g., in less than $1\ \mu\text{sec}$, as is achievable in pulse radiolysis, the transient free radicals and their kinetics can be easily studied and accurately measured. The kinetic parameters are usually derived from the kinetics of the formation of $\text{HArO}\cdot$ which usually has a strong absorption spectrum in the visible region (see the section on spectral properties).

REACTIVITIES OF PEROXY RADICALS

The reactivity of an antioxidant will not be the same for all peroxy radicals. From Table 7.1 it is clear that chlorinated peroxy radicals have considerably higher reactivities. The effect of chlorination can be explained by the strong electron-withdrawing properties of halogen atoms:

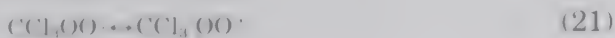


TABLE 7.1. HYDROGEN ATOM ABSTRACTION RATE CONSTANTS^a FOR $c\text{-C}_6\text{H}_{11}\text{OO}\cdot$ AND $\text{CCl}_3\text{OO}\cdot$ REACTING WITH ANTIOXIDANTS, HArOH ^b

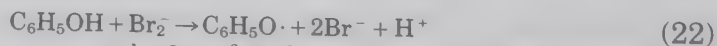
HArOH	$\text{CCl}_3\text{OO}\cdot^c$	$c\text{-C}_6\text{H}_{11}\text{OO}\cdot^c$
α -Tocopherol	200 ± 20	5.7 ± 1
γ -Tocopherol	160 ± 20	5.7 ± 1
2,2,5,7,8-Pentamethyl-6-hydroxychroman	129 ± 8	6.8 ± 0.5
2,5,7,8-Tetramethyl-2-hydroxymethyl-6-hydroxychroman	88 ± 5	7.0 ± 0.5
3,3,5,7,8-Pentamethylchromanol-S analog	113 ± 8	4.1 ± 0.2
N-Ethyl-1,2,3,4-tetrahydro-5,7,8-trimethyl-6-hydroxyquinoline	258 ± 20	13.2 ± 1.6
BHA	22.6 ± 1.5	2.6 ± 0.3
3-BHA	24 ± 3.1	—
4-Methoxyphenol	6.8 ± 0.3	<0.5
4- <i>t</i> -Butylphenol	0.63 ± 0.1	<0.5
3,5-di- <i>t</i> -Butylphenol	1.0 ± 0.1	<0.5
Pentamethylphenol	7.6 ± 1.0	<0.5
1-Naphthol	10 ± 2	<0.5

^a Rate constants in units of $10^6 M^{-1} \text{sec}^{-1}$.^b Data reported in Simic and Hunter (1984B).^c All reactions run in air saturated CCl_4 or $c\text{-C}_6\text{H}_{12}$.

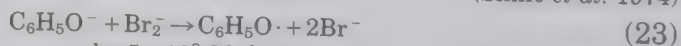
The effect is confined to the α position of the peroxy radical. It can be exerted by other electron-withdrawing groups. The decrease in number of Cl atoms in the α position results in decreased reactivities. These effects are shown in Table 7.2.

REACTIVITIES OF ANTIOXIDANTS

Reaction rate constants for various antioxidants with peroxy radicals are shown in Table 7.1. The reactivity of an antioxidant is greatly increased by ionization of the hydroxy group. For example, in aqueous solution,



$$k = 6 \times 10^6 M^{-1} \text{sec}^{-1} \quad (\text{Simic et al. 1974})$$



$$k = 5 \times 10^8 M^{-1} \text{sec}^{-1} \quad (\text{Simic et al. 1974})$$

Reactivities of some ionized (deprotonated) hydroxy aromatic derivatives, HArO^- , are shown in Table 7.3.

TABLE 7.2. RATE CONSTANTS^a FOR REACTION OF CHLORINATED PEROXY RADICALS WITH PHENOL AND TYROSINE (pH = 12)^b

Radical	Phenol	Tyrosine
Cl ₃ COO·	230 ± 20	130 ± 20
Cl ₂ CHOO·	110 ± 10	100 ± 10
ClCH ₂ OO·	11 ± 1	21 ± 1
·OOCCL ₂ CO ₂ ⁻	14 ± 1	16 ± 2
·OOCHClCO ₂ ⁻	7.1 ± 1	12 ± 1
·OOCF ₂ CO ₂ ⁻	15 ± 1	30 ± 3
CH ₃ OO·	<1	<1

^a Rate constants in units of 10⁶ M⁻¹ sec⁻¹.^b Data taken from Packer *et al.* (1980).TABLE 7.3. RATE CONSTANTS FOR CO₃⁻ REACTING WITH PHENOLIC ANTIOXIDANTS (ANIONIC FORMS, HArO⁻) AT pH = 12^a

Phenol	4.7 × 10 ⁸
1-Naphthol	3.1 × 10 ⁹
2-Naphthol	1.3 × 10 ⁹
2,5,7,8-Tetramethyl-2 carboxy-6-hydroxychroman	1.5 × 10 ⁹
2,5,7,8-Tetramethyl-2-hydroxy-methyl-6-hydroxychroman	2.2 × 10 ⁹

^a Data from Simic and Hunter (1984A).

SPECTRAL PROPERTIES

Generally, alkyl peroxy radicals have rather broad, featureless absorption spectra with maxima about 250 nm having extinction coefficients around 1500 M⁻¹ cm⁻¹ (McCarthy and MacLachlan 1961; Simic and Hayon 1971). Most phenoxy radicals exhibit broad absorptions around 340 nm and somewhat sharper peaks around 400–425 nm (Steenken and Neta 1982). The exceptions are the S- and N-analogs of the tocopherols (the fifth and sixth entries in Table 7.1), which show broad peaks around 500 nm. Figure 7.1 shows the more typical aroxy radical spectrum. The extinction coefficient at 403 nm was also checked in aqueous carbonate solutions, where the *G* value for aroxy radical formation is most reliable.



ESR spectra of aroxy radicals have been reported (Becconsall *et al.* 1960) which show structure indicating that the unpaired electron is substantially delocalized into the aromatic ring.

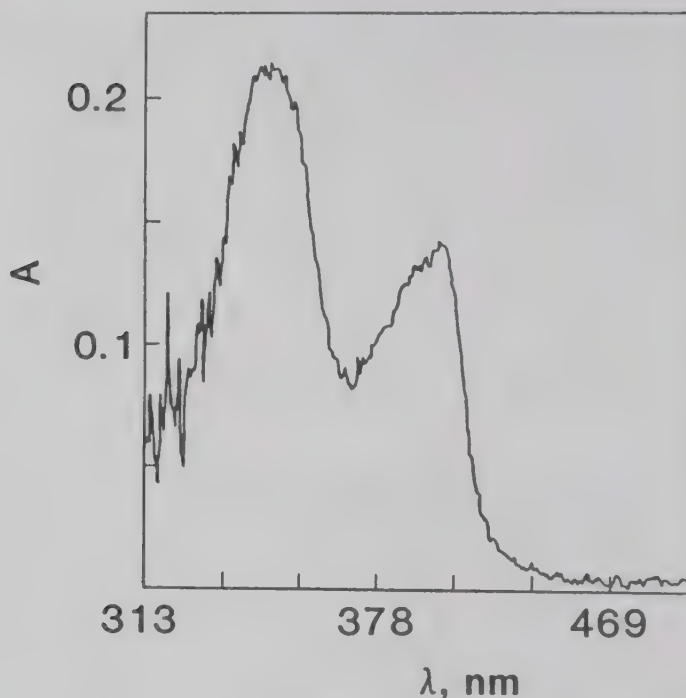
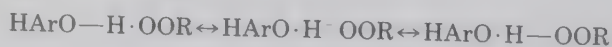


FIG. 7.1. Transient spectra of the aroxy radical formed by H-atom abstraction from BHA by $\text{CCl}_3\text{OO}\cdot$. Conditions: 2.0 mM BHA in air-saturated CCl_4 ; dose, 53 Gy/pulse, 22°C .

STRUCTURE-REACTIVITY RELATIONSHIPS FOR ANTIOXIDANTS

The reactivity of phenolic antioxidants toward a given peroxy radical has been found to correlate with the electrophilic effects of ring substituents. Thus, in reactions of styryl peroxy and tetralyl peroxy (Howard and Ingold 1963; 1965) radicals with phenols having different *p*-substituents but otherwise identical structures, a correlation between k_5 and σ^+ (Brown's electrophilic substituent values) existed. The reaction constants obtained from this correlation indicated that the transition state complex consisted of a dipolar resonance structure where a formal positive charge resides on the phenol O atom and whose importance increases with increasing electron-releasing power of the *p*-substituent.



Accordingly, phenols having *p*-alkoxy groups (a good electron-releasing group) should be more reactive than an unsubstituted or alkyl-

substituted phenol. From Table 7.1, 4-methoxyphenol reacts about 10 times faster than 4-*t*-butylphenol with $\text{CCl}_3\text{OO}\cdot$. Other reactivity-increasing structural features include alkyl substitution in the 2, 3, 5, and 6 positions, but with the restriction that the ortho groups do not sterically shield the phenolic group.

It should be noted that the 6-hydroxychromans are considerably more reactive toward peroxy radicals than are the simple 4-alkoxyphenols. This rate enhancement has been ascribed to a steric restriction due to the saturated ring which holds the alkoxy O atom so that its *p* orbitals are parallel to the *p* orbitals of the aromatic system (Burton and Ingold 1981). Evidently, the same considerations apply when the hetero atom is S or N.

CONCLUSIONS

Design of a new antioxidant cannot be based on the classical assessments of the antioxidizing efficiencies of a few known antioxidants. It has to be based on the structure-reactivity relationship derived through kinetic studies as presented here. Second, the lipophilicity of the antioxidant has to be adjusted in order to concentrate the antioxidants in the regions of intensive autoxidation. In most foods the major problems stem from autoxidation of bulk fats, and the antioxidants used for that purpose are highly lipophilic. However, there are other loci of autoxidation which contribute toward deterioration and spoilage of foods. Processes falling into this category, e.g., autoxidation of membranes, have received much less attention, and the design of an antioxidant would require mechanistic understanding of autoxidation within the biochemical framework. It is hoped that new classes of antioxidants will be developed to respond to these nonclassic autoxidation processes and that kinetic studies by pulse radiolysis would facilitate the accomplishment of those goals.

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Mechanisms of Oxidoreductases Important in Food Component Modification

*John R. Whitaker*¹

INTRODUCTION

Six types of chemical reactions are catalyzed by enzymes; these are oxidoreduction, transfer, hydrolysis, making or adding to double bonds (lyases), isomerization, and bond formation requiring high-energy phosphates (ligases). While all of these reactions are important in food production, the food scientist is especially concerned with oxidoreduction and hydrolysis reactions. In general, much more emphasis is placed on the hydrolytic reactions than on the oxidoreductions, perhaps because hydrolytic reactions are so important in malting and brewing, in cheese production, in conversion of starch to glucose and fructose, and in human metabolism.

In this chapter, I have singled out several oxidoreductase enzymes important to the food scientist and have provided some data on the mechanism of action of each of these enzymes. In most cases there is a wealth of data on each enzyme and several mechanisms have been proposed. I have selected in each case the mechanism that appears to explain as much of the experimental data as possible. Mechanisms

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have a way of becoming obsolete rapidly as new methods, new instrumentation, and new concepts develop. Therefore, I offer only as a disclaimer that I have done the best I can at this moment in the state of knowledge of each enzyme.

The enzymes selected are as follows:

1. *Polyphenol oxidase*, a copper-containing oxidase and one of the most damaging enzymes to quality maintenance of fresh fruits, particularly. However, the beneficial effects of polyphenol oxidase in tea, coffee, cocoa, prunes, raisins (dark), figs, etc. are obvious.
2. *Lipoxygenase*, nonheme iron-containing enzyme responsible for oxidation of the essential fatty acids linoleic, linolenic, and arachidonic acids, the development of off-flavors especially in soybeans, green peas, and green beans, and secondary free radical oxidation of carotenes and other pigments.
3. *Peroxidase and catalase*, heme iron-containing enzymes which are widely used to determine the adequacy of blanching of fruits and vegetables prior to freezing and storage. Catalase is used in conjunction with glucose oxidase in removal of O_2 from head spaces of packages of foods. Peroxidases are used along with glucose oxidase to determine glucose concentrations of biological materials.
4. *Xanthine oxidase*, a molybdenum-containing enzyme that oxidizes purines formed from nucleic acid metabolism to uric acid, which may be an important factor in gout. Active xanthine oxidase left in high-temperature-short-time (HTST) pasteurized milk has received a lot of unsubstantiated notoriety as a potential oxidant in blood should it pass the digestive system and the small intestinal mucosa still in an active state.
5. *Glucose oxidase*, a flavin adenine dinucleotide (FAD)-containing enzyme that is quite specific for β -D-glucose and therefore of immense value in rapidly determining glucose concentration in biological materials, in conjunction with peroxidase. Glucose oxidase is used for removal of glucose from dried egg whites to prevent the Maillard reaction and in removal of O_2 from head space of packages of dried foods, wines, etc. (in conjunction with catalase).
6. *Alcohol dehydrogenase and aldehyde dehydrogenase*, NAD^+ / $NADH$ -requiring enzymes, that reduce aldehydes to alcohols or oxidize aldehydes to acids. These reactions are important in reducing the levels of undesirable aldehydes and associated odors from soybean flour, for example, and in the development of esters, major flavor compounds in some fruits.

POLYPHENOL OXIDASE

Introduction

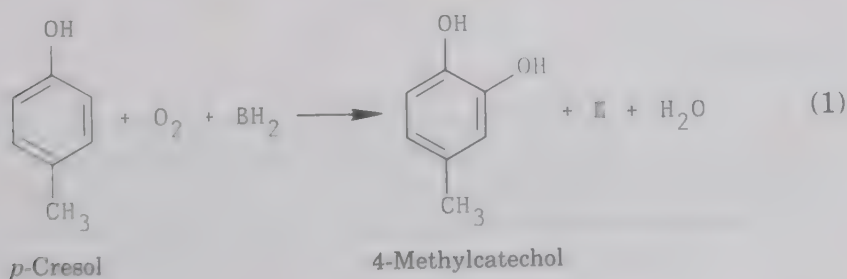
Polyphenol oxidase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase; EC 1.14.18.1; 1,2-benzenediol:oxygen oxidoreductase, EC 1.10.3.1) is probably found in most plant tissues, but especially in high concentrations in mushrooms, potato tubers, peaches, apples, bananas, avocados, tea leaves, coffee beans, and tobacco leaves. The activity can vary markedly in different varieties of the same plant, in different stages of maturity, cultivation conditions, etc.

Polyphenol oxidase is of particular importance in food science and technology because of the brown (usually), red, and blue discolorations of bruised fruit and vegetables resulting from the action of this enzyme. Estimates of up to 50% loss of fresh tropical fruits worldwide can occur due to this enzyme. Not only is there undesirable color formation, but browning results in loss of nutrient quality and undesirable taste. On the other hand, polyphenol oxidase browning is a desired activity in tea, coffee, cocoa, prunes, dates, and dark raisin production.

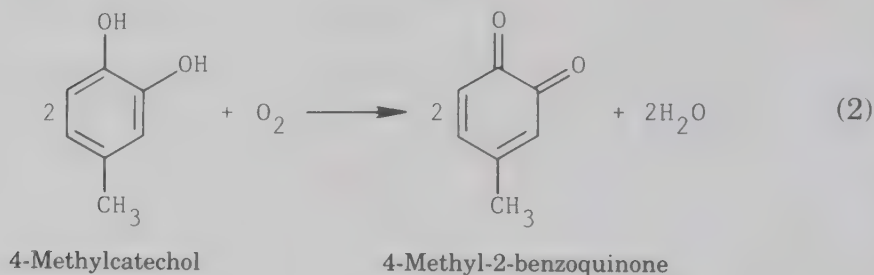
Because of the broad substrate specificity of polyphenol oxidase, it has been called tyrosinase, polyphenolase, phenolase, catechol oxidase, catecholase, and cresolase at various times. The enzyme occurs in animals where the major substrate is tyrosine; this enzyme, with a more narrow specificity range than the enzyme in plants, is responsible for skin color, including freckles (concentration of melanins).

Reactions Catalyzed

Some polyphenol oxidases catalyze two quite different types of reactions, the hydroxylation of monophenols to form *o*-dihydroxyphenols (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1; monophenol monooxygenase; cresolase-type activity; Eq. 1)



and the further oxidation of *o*-dihydroxyphenols to benzoquinone (1,2-benzenediol:oxygen oxidoreductase; EC 1.10.3.1; catecholase-type activity; Eq. 2).



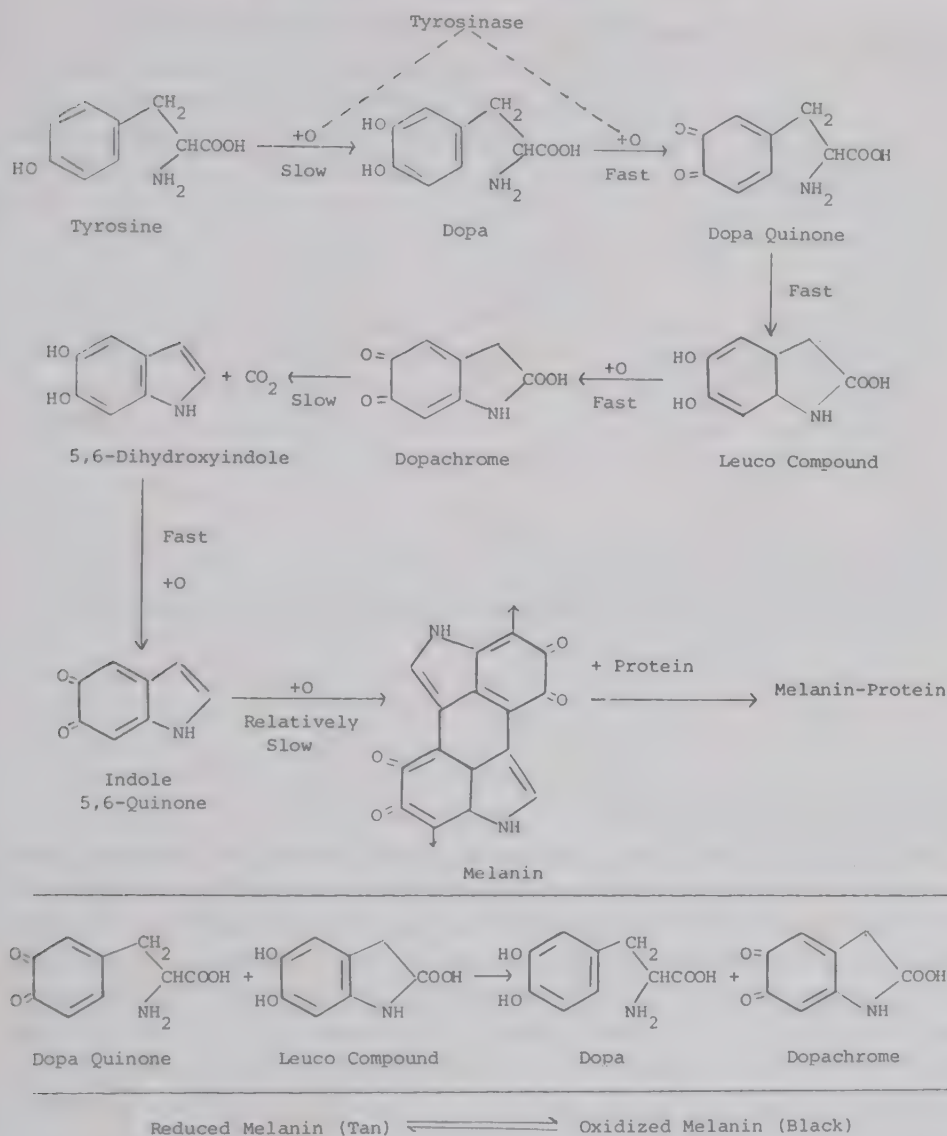
All polyphenol oxidases have activity on *o*-diphenols. Polyphenol oxidases from bananas, tea leaf, tobacco leaf, and clingstone peach have been reported to oxidize *o*-diphenols only while the enzyme(s) from potato, apple, sugar beet leaf, broad bean leaf, mushrooms, and *Neurospora crassa* have both types of activity.

Unfortunately, for assay methods, oxidation of *o*-diphenols does not stop at the level of *o*-benzoquinone. The *o*-benzoquinone is quite reactive with O_2 nonenzymatically and continues to undergo oxidation resulting in melanin (Scheme 1). Also, kinetic studies of polyphenol oxidase are complicated by substrate $\rightarrow$ product inactivation (see section on kinetics and mechanism, p. 126).

The initial velocity of reaction of polyphenol oxidase with monophenols is quite slow and shows a lag period in the absence of catalytic amounts of BH_2 (Eq. 1). Addition of as little as $1 \times 10^{-7} M$ catechol to the reaction abolishes the lag period, giving normal initial reaction rate kinetics. Since the enzyme can be saturated with BH_2 (example, catechol), there appears to be a binding site for this *o*-diphenol in the active site. Its role in the mechanism of polyphenol oxidase is poorly understood. A possible explanation will be offered below.

Specificity

The substrate specificity of plant polyphenol oxidases is quite broad, and there are individual differences among polyphenol oxidases from different sources (Table 8.1). Note particularly the quite different relative specificities of the enzyme from potato, peach, and broad bean leaf on chlorogenic acid and caffeic acid compared to similar relative



(From Lerner 1953)

SCHEME 1

activities on 3,4-dihydroxy-L-phenylalanine. The activities of potato and broad bean leaf enzymes have low activities on *p*-cresol (monophenol) compared to catechol (*o*-diphenol), and peach enzyme has no activity on monophenols.

TABLE 8.1. RELATIVE SUBSTRATE SPECIFICITIES OF THREE POLYPHENOL OXIDASES

Substrate	Activity relative to catechol		
	Potato ^a	Peach ^b	Broad bean leaf ^c
Di- or triphenolic compounds			
Catechol	100	100	100
4-Methylcatechol		51.5	200–225
<i>d</i> -Catechin		31.8	
Chlorogenic acid	140	22.2	8
Caffeic acid	76.5	0	12.5
Protocatechuic acid		16.3	0.11
3,4-Dihydroxy-L-phenylalanine	54.3	40.5	50
Dopamine		45.6	
Gallic acid		25.7	0.22
Pyrogallol			85–95
Monophenolic compounds			
<i>p</i> -Cresol	5.5	0	4
<i>p</i> -Coumaric acid	Nil	0	0.05

^a From Macrae and Duggleby (1968); pH 7.0.

^b From Wong *et al.* (1971); for component A of clingstone peach at pH 6.8 and 30°C.

^c From Robb *et al.* (1966).

Kinetics and Mechanism

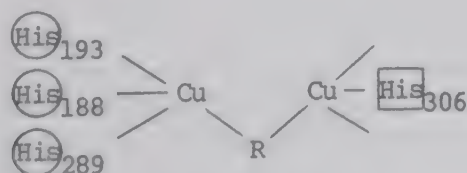
Polyphenol oxidases are copper-containing enzymes. This is shown by removal of the copper by treatment of the *N. crassa* enzyme with 100 mM KCN for 2 hr, followed by gel filtration on Sephadex G-25 in 50 mM sodium phosphate, pH 7.5, for example (Pfiffner *et al.* 1981). The apoenzyme has no catalytic activity, but can be reactivated by incubating the apoenzyme with 20-fold excess of CuSO₄ for 20 hr at 4°C (Pfiffner *et al.* 1981). Near stoichiometric amounts of copper are found in polyphenol oxidases from *N. crassa* and mushroom by atomic absorbance spectrometry.

Most of the early kinetic and mechanistic studies were done on mushroom polyphenol oxidases. However, preparation of pure mushroom polyphenol oxidase in sufficient amounts for such studies is complicated by the presence of at least four isozymes which have variable ratios of activity on *p*-cresol (monophenol) and catechol (*o*-diphenol) and contain variable ratios of Cu(I) and Cu(II) (Bouchilloux *et al.* 1963). The subunit weight of mushroom polyphenol oxidase is controversial. It is generally assumed to be composed of at least four subunits (128,000 MW with 32,000 MW subunits), each with an active site. Strothkamp *et al.* (1976) suggested a model of two large subunits (H) of 45,000 and two small subunits (L) of 13,000. Golan-Gold-

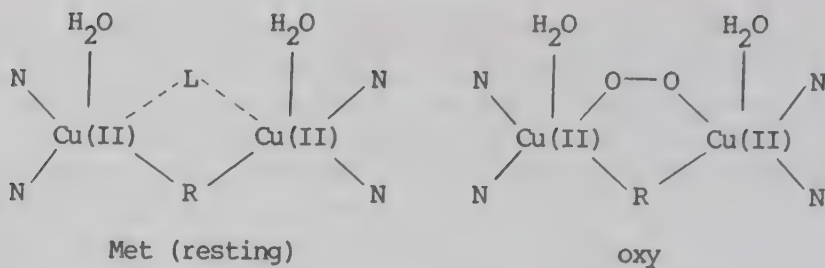
hirsh and Whitaker (1984A) reported SDS-PAGE data indicating subunit weights of 105,000 (MW), 53,000, and 28,000.

In contrast, *N. crassa* polyphenol oxidase has a single subunit of 46,000 MW (Lerch 1978) and contains a single binuclear copper active site (Deinum *et al.* 1976) which, when in the reduced state, binds O_2 reversibly (Lerch 1976). There is also great similarity between *N. crassa* polyphenol oxidase and hemocyanins in the nature of the binuclear copper active site (Himmelwright *et al.* 1980). Therefore, more rapid progress has been made in working out the chemistry of the active site of this enzyme than for mushroom polyphenol oxidase. Based on present knowledge, the active sites and chemistry are likely to be quite similar for the two enzymes. Unless specified, the following discussion of chemistry and mechanism will concentrate on data for the *Neurospora* enzyme.

The active site of *Neurospora* polyphenol oxidase contains two copper atoms which cycle between the one and two oxidation states during the reaction. The two coppers are in proximity to each other (3.5–5.0 Å). One is coordinated to three histidine residues (Nos. 193, 188, and 289) of the protein, based on dye-sensitized photooxidation, and the other copper is coordinated to another histidine residue (No. 306) based on reaction inactivation studies (Pfiffner *et al.* 1981) (Scheme 2). Fry and Strothkamp (1983) have recently shown that four histidine residues on the heavy subunit of mushroom polyphenol oxidase are destroyed by irradiation in the presence of citrate (pH 5.6, with 300–420 nm light), resulting in the loss of both monophenol and *o*-diphenol activities. The photoactivated oxidation, which does not require O_2 , may involve generation of a free radical from citrate which then attacks nearby histidine residues. In Schemes 2 and 3, the two coppers are held adjacent to each other (3.5–5.0 Å) by an endogenous bridging ligand (R; either phenolate or oxo) which provides a super exchange pathway for antiferromagnetic coupling, resulting in the absence of a detectable EPR signal (Himmelwright *et al.* 1980). In the oxy enzyme (Scheme 3), the two coppers are also bridged by a peroxide. In the resting enzyme (Met), the second ligand can be other substances including NO_2^- , N_3^- , etc. (Scheme 3; Himmelwright *et al.* 1980).



SCHEME 2



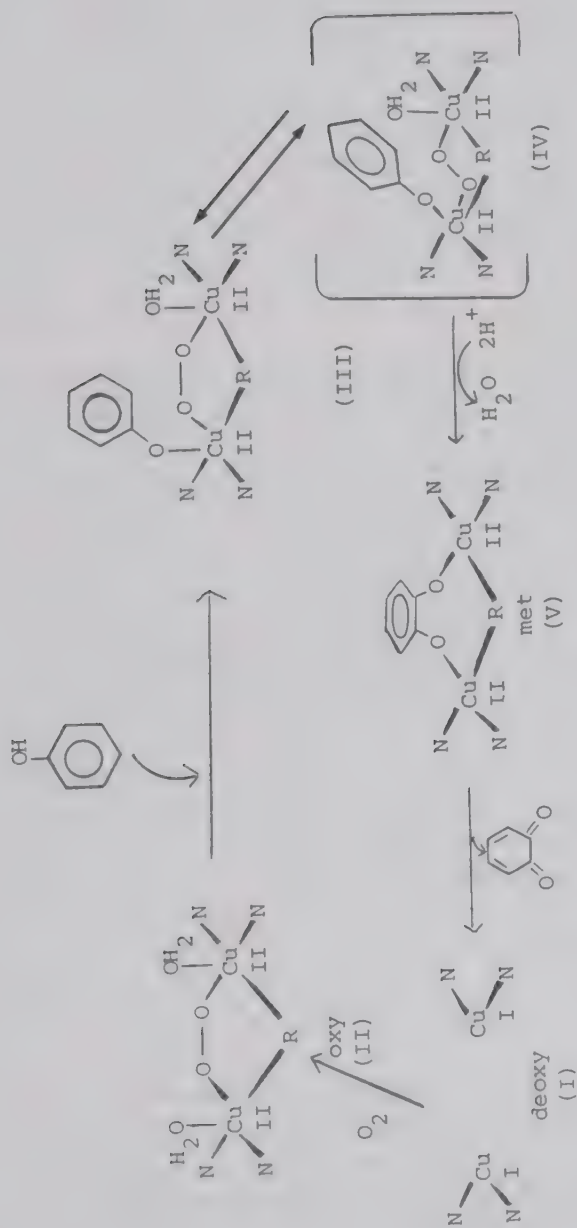
SCHEME 3

Based on the presently available data, Winkler *et al.* (1981) have proposed the following scheme for the hydroxylation and oxidation of monophenols and *o*-diphenols, respectively, by *Neurospora* polyphenol oxidase (Scheme 4).

At the end of a reaction the active site is in the deoxy form (I). It may either bind O_2 to form the oxy form (II) or possibly go to the Met form (Scheme 3), which may require a catalytic amount of an *o*-diphenol to reactivate to the deoxy form (not shown in Scheme 4; see Eq. 1 above). The oxy form (II) binds a monophenol to produce form (III) where the hydroxyl group of the monophenol has replaced one of the H_2O molecules on one of the $Cu(II)$ (form III). Through rearrangement to a trigonal-bipyramidal intermediate (form IV), the monophenol may labilize the peroxide from one copper, leaving a reactive polarized peroxide which can hydroxylate the phenol. Following coordination of the two *o*-hydroxyl groups of the resulting diphenol to both $Cu(II)$ (form V), the *o*-diphenol then is oxidized to *o*-benzoquinone while the two $Cu(II)$ are reduced to two $Cu(I)$ (form I). The benzoquinone then dissociates from the enzyme which can recycle in the process. In those polyphenol oxidases which cannot perform hydroxylation of monophenols, the system would presumably cycle through forms $I \rightarrow II \rightarrow V \rightarrow I$.

Reaction Inactivation

Polyphenol oxidase undergoes rapid reaction inactivation. About one in 5000–10,000 cycles results in inactivation of a molecule of enzyme (Golan-Goldhirsh and Whitaker 1984A). Wood and Ingraham (1965) proposed that this inactivation might be due to reaction of benzoquinone with an amino group in or near the active site. More recently, *Neurospora* polyphenol oxidase (Pfiffner *et al.* 1981) and mushroom



SCHEME 4

polyphenol oxidase (Golan-Goldhirsh and Whitaker 1984B) have been shown not to covalently bind benzoquinone during reaction inactivation. Rather, there is a loss of His-306 in *Neurospora* polyphenol oxidase, which results in a corresponding loss of one copper atom and loss of activity (Pfiffner *et al.* 1981). A likely candidate for this reaction inactivation is form IV of Scheme 4, where the peroxide ligand is presumed to form a polarized peroxide which hydroxylates the monophenol. Every so often, there could be an attack of this highly reactive peroxide on His-306, resulting in inactivation. Further data to support this hypothesis are needed. Golan-Goldhirsh and Whitaker (1984B) have shown that ascorbic acid and Cu^{2+} efficiently oxidize all four histidines of the active site of mushroom polyphenol oxidase (pH 6.5, 25°C, 20 hr).

Polyphenol oxidase activity in fruits and vegetables can be controlled by addition of sulfite, ascorbic acid, and thiol compounds (Golan-Goldhirsh and Whitaker 1984C). In part, this is due to reduction of the *o*-benzoquinone to *o*-diphenol, preventing color formation; in part it is due to a direct effect of these compounds on the enzyme (Golan-Goldhirsh and Whitaker, 1984C). Now that the chemistry of the active site is known, perhaps the cause of the direct effect of these compounds in inactivating polyphenol oxidase can be determined. Also, it is hoped that one can enhance the reaction inactivation of polyphenol oxidase for rapid and specific (k_{cat} inactivation) control of enzymatic browning.

Steady-state kinetic studies of polyphenol oxidase indicate that it probably follows an ordered bi-bi mechanism in which the O_2 must add first (see Scheme 4) followed by the mono- or *o*-diphenol (Ingraham 1957; Rivas and Whitaker 1973).

LIPOXYGENASE

Introduction

Lipoxygenase (linoleate:oxygen oxidoreductase; EC 1.13.11.12) is found in a wide variety of plants, particularly the legumes. It has been reported in alfalfa, peas, beans, peanuts, potatoes, and radishes, among others. The highest concentration of lipoxygenase has been reported in soybeans; urd beans, mung beans and peas contain about 50% as much lipoxygenase, while peanuts and wheat contain 1–2% of that found in soybeans (Siddiqi and Tappel 1957).

Lipoxygenase is of particular importance to scientists in food science and technology because of its destruction of the essential fatty acids, linoleic, linolenic, and arachadonic acids, which result in the

development of off-flavor and odor, characterized as haylike flavors in underblanched green peas and green beans and beany flavor in soybeans and soybean products. The intermediate free radical hydroperoxides produce damage to other compounds, including carotene, vitamin A, and proteins. Lipoxygenase is involved in flavor formation in plant materials, especially in tomatoes due to the formation of 2-hexenal. The aldehydes formed can be oxidized further by aldehyde dehydrogenase or aldehyde oxidase to carboxylic acids or reduced by alcohol dehydrogenase to alcohols. The carboxylic acids and alcohols then nonenzymatically form esters with characteristic odors.

General Properties

The best described lipoxygenase is lipoxygenase-1 from soybean which was crystallized in 1947 (Holman 1947; Theorell *et al.* 1947). The enzyme has a molecular weight of 102,000 [more recently reported to be 98,500 (Vliegthart *et al.* 1979)] with an isoelectric point of 5.4 and a single polypeptide chain containing one iron atom involved in the active site (Chan 1973; Roza and Francke 1973; Pistorius and Axelrod 1973; Pistorius *et al.* 1976). The pH optimum is 8–9, depending on the presence or absence of detergents added to solubilize the substrates. Soybeans contain three other isoenzymes, all in lesser amounts and with pH optima near pH 6.5. Unless specified, the following discussion is for lipoxygenase-1.

Mechanism

Lipoxygenase belongs to the dioxygenase group of enzymes in which both atoms of O_2 are incorporated into the product. It oxidizes polyunsaturated fatty acids and esters (at a much lower rate) containing a *cis,cis*-1,4-pentadiene system (Fig. 8.1) to form hydroperoxides as the primary products as well as a number of secondary products (Hamberg and Samuelsson 1967).

The reaction is shown in more detail in Fig. 8.2 for 8,11,14-(*all-cis*)eicosatrienoic acid. The sequence of chemical events, all done while the enzyme is attached, are as follows: (1) The enzyme forms a stereospecific complex with the unsaturated fatty acid; (2) soybean lipoxygenase-1 removes only the H_L (Pro-S) hydrogen from the ω -8 (C-13) carbon (Figs. 8.1 and 8.2) while corn germ lipoxygenase removes the H_R (Pro-R) hydrogen (Egmond *et al.* 1972); (3) the fatty acid free radical isomerizes to place the unshared electron at ω -6 (C-15), causing conjugation and *cis*→*trans* isomerization of the double bond; (4) the O_2 , bound at this point to enzyme or earlier (see later on), adds to the

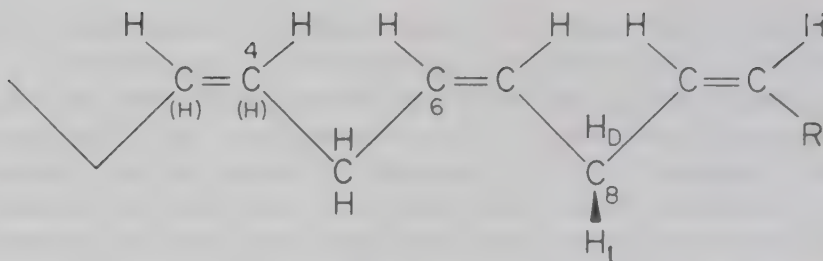
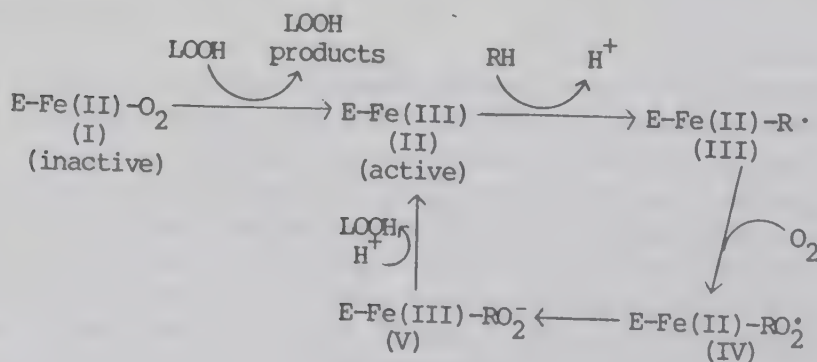


FIG. 8.1. The essential part of a fatty acid which permits it to be a substrate for lipoxygenase. Required are *cis* double bonds at ω -6 and ω -9 positions, a hydrogen in the L_S position on ω -8 carbon, and hydrogen at the ω -6 carbon. The ω -3 and ω -4 positions may be saturated or unsaturated.

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fatty acid radical at ω -6 (C-15) to give the L_S -hydroperoxy radical in the case of soybean lipoxygenase-1 or ω -10 (C-11) in the case of corn germ (Gardner and Weisleder 1970) and potato (Galliard and Phillips 1971) lipoxygenases. Therefore, the O_2 must enter from the opposite side of the molecule from which the hydrogen is removed in order to produce the *trans*- L_S -hydroperoxy radical in the case of soybean lipoxygenase-1; (5) an electron abstracted from the enzyme and a proton from the medium give the product 15- L_S -hydroperoxy-8,11,13-*cis,cis,trans*-eicosatrienoic acid from 8,11,14-(*all-cis*)eicosatrienoic acid. The corn germ lipoxygenase gives the 9- D_R -hydroperoxide in the case of linoleic acid.

The 15- L_S -hydroperoxy-8,11,13-*cis,cis,trans*-eicosatrienoic acid dissociates from the enzyme under aerobic conditions to regenerate the enzyme (de Groot *et al.* 1975) (see Scheme 5), but under anaerobic conditions it is further catalyzed to a variety of products including dimers, oxodienoic acids, and *n*-pentane (Galliard 1975). This reac-



SCHEME 5

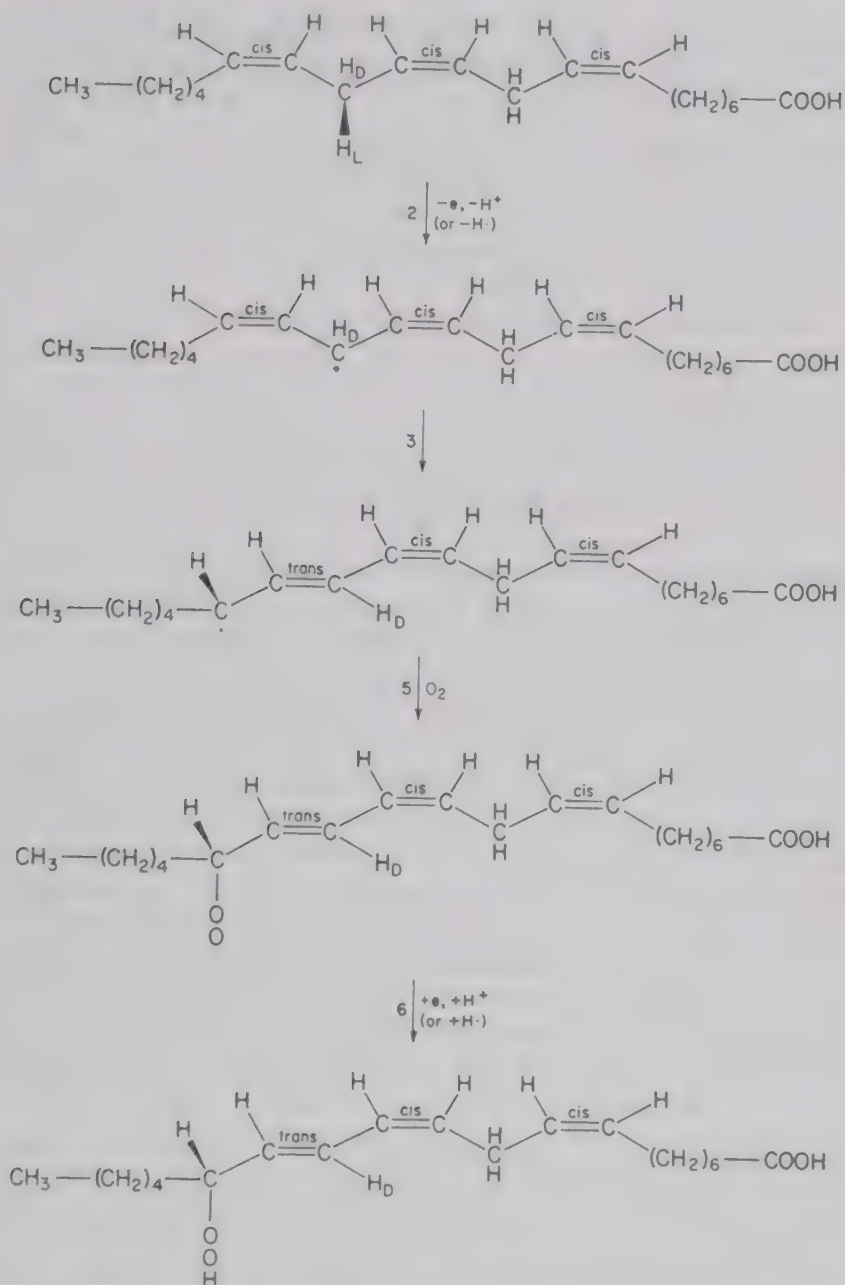


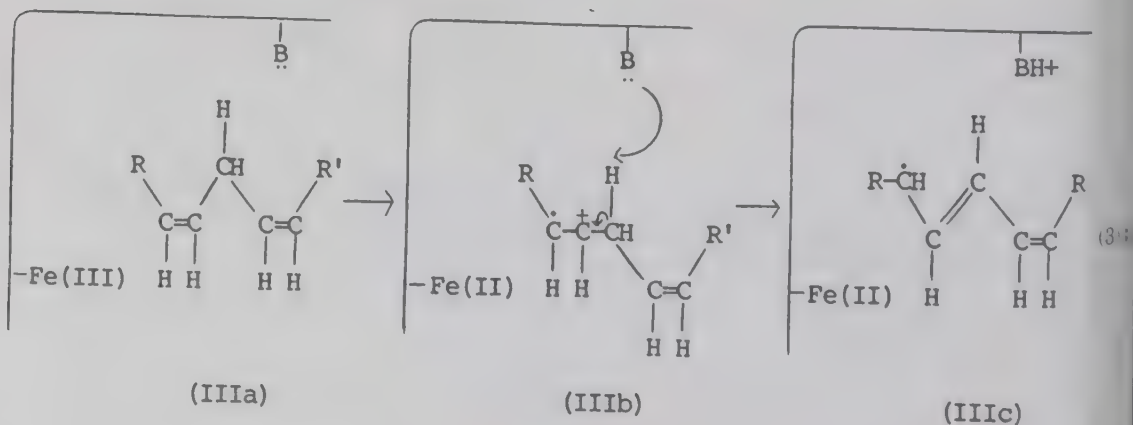
FIG. 8.2. Reaction catalyzed by lipoxygenase, using 8,11,14-*cis,cis,cis*-eicosatrienoic acid as a typical substrate. See text for details.

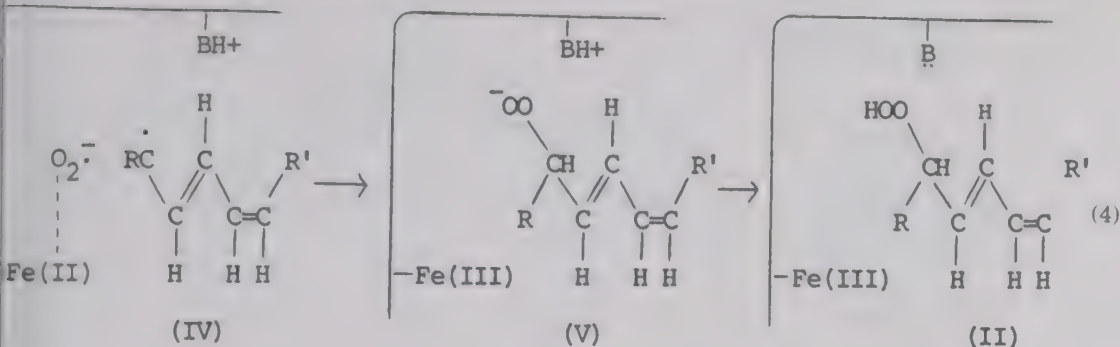
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tion, which has been studied extensively by adding the fatty acid hydroperoxide to lipoxygenase directly, also is catalyzed by the enzyme under aerobic conditions (de Groot *et al.* 1975), but at a rate approximately one thousandth that under anaerobic conditions (Aoshima *et al.* 1981). Under anaerobic conditions the rate of catalysis of linoleic acid hydroperoxide to further products was shown to be approximately the same as the rate for formation of the linoleic acid hydroperoxide (Pistorius *et al.* 1976).

The iron atom plays a key role in the oxidation of the polyunsaturated fatty acid as shown by absorption, fluorescence, and spin resonance spectroscopy (Pistorius *et al.* 1976). The nonheme iron atom is tightly bound to lipoxygenase, liganded in part to a sulfur, but the other ligands are not known. The mechanism proposed by de Groot *et al.* (1975) appears to best incorporate all available data for the reaction (Scheme 5).

The resting form of the enzyme (I) has been shown to be E-Fe(II)-O₂ (Vliegthart *et al.* 1979) by electron paramagnetic resonance studies of the NO complex of the enzyme. On adding substrate to the resting form of the enzyme, there is a lag period which can be abolished by adding the product linoleic acid hydroperoxide (Haining and Axelrod 1958), presumably resulting in the formation of the active enzyme, E-Fe(III) (II). E-Fe(III) removes the hydrogen stereospecifically from the ω -8 position of the polyunsaturated fatty acid (Fig. 8.1) to give the E-Fe(II)-R $\cdot$ free radical (III). Based on reactions with model compounds and from known chemical reactions, Gibian and Galaway (1977) proposed that this chemical reaction could proceed in two steps, as shown by Eq. 3 (intermediates IIIa,b,c). Conversion of IIIb to IIIc is thought to be the rate-determining step. O₂ then binds to the enzyme and forms product IV which is the fatty acid hydroperoxide radical, E-Fe(II)-RO₂ $\cdot$. Gibian and Galaway (1977) have proposed the





chemistry could be that shown in Eq. 4 using the E-Fe-(III)-RO₂· form of the enzyme; the present author has used the E-Fe(II)-RO₂· form in accordance with the postulation of de Groot *et al.* (1975). The Fe(II) can then serve as a source of the electron to form intermediate V which then is protonated by the acid group -BH⁺ on the enzyme to release the LOOH, regenerating the active form of the enzyme E-Fe(III) (species II) for continued catalysis. Once E-Fe(II)-O₂ is activated to E-Fe(III), catalytic amounts of LOOH are no longer needed as long as substrate is present.

Much of the above mechanism, particularly the chemistry, is still speculative. Several uncertainties still exist in the mechanism. These are the following:

1. How is the iron atom liganded to the protein and what is its spin state? Pistorius *et al.* (1976) concluded that the electron spin resonance observed with soybean lipoxygenase-1 in the presence of linoleic acid peroxide is similar to that observed for heme proteins in the high-spin ferric form and for the nonheme enzyme, protocatechuate 3,4-dioxygenase.

2. Why does there appear to be two K_m values for some lipoxygenases? Initial reaction velocities on soybean lipoxygenase at sodium linoleate concentrations below $3 \times 10^{-4} M$ at pH 9.0 and 20°C without addition of a solubilizer gave a K_m for linoleate of $2 \times 10^{-5} M$ (Tappel *et al.* 1952; Allen 1968). This K_m value is almost at the half-way point of the critical micelle concentration of linoleic acid (Chen 1984). As the concentration of linoleic acid was increased above $3 \times 10^{-4} M$ in the presence of a constant ratio of Tween-20, the velocity of the lipoxygenase-catalyzed reaction increased substantially, and the data around $10^{-3} M$ substrate gave excellent Lineweaver-Burk plots with a K_m value of $1.63 \times 10^{-3} M$ (Chen 1984). At these higher concentrations of linoleic acid, the K_m for O₂ was $1.18 \times 10^{-4} M$ compared to $3.0 \times 10^{-5} M$ at $3.6 \times 10^{-4} M$ linoleic acid (Tappel *et al.* 1952).

TABLE 8.2. SUBSTRATE SPECIFICITY OF PURIFIED PEA AND SOYBEAN LIPOXYGENASES

Substrate	Activity relative to linoleic acid ^a	
	Pea lipoxygenase ^b	Soybean lipoxygenase ^c
Linoleic acid	100	100
Linoleyl alcohol	69.3	0.92
Linoleyl acetate	28.4	0.48
Methyl linoleate	34.1	0.63
Ethyl linoleate	18.4	0.35
Propyl linoleate	21.9	0.96
Trilinolein	77.2	1.27

Source: Chen (1984).

^a Substrate was made up in ethanol; final concentration in reaction was 1.90%.

^b Purified from fresh English peas.

^c Commercially available enzyme.

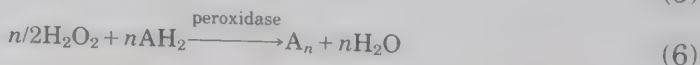
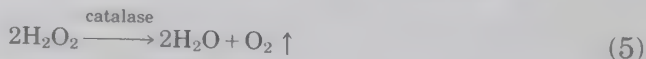
3. Why, at lower substrate concentrations (below $3 \times 10^{-4} M$), do the kinetics appear to follow a ping-pong bi-bi mechanism with double substrate inhibition (Verhagen *et al.* 1978) while at higher substrate concentrations ($>3 \times 10^{-4} M$), the kinetics indicate a sequential mechanism (Chen 1984)?

4. Why does soybean lipoxygenase-1 have much better activity on underivatized fatty acid substrates (Chen 1984), while soybean lipoxygenase-2 and pea lipoxygenase have nearly equal activities on derivatized and free fatty acid substrates (Table 8.2)? Since the lipoxygenases presumably recognize the ω end of the substrate (end opposite the carboxyl group), why the difference?

PEROXIDASE AND CATALASE

Introduction

Peroxidase (donor:hydrogen-peroxide oxidoreductase; EC 1.11.1.7) and catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase; EC 1.11.1.6) differ markedly in their protein characteristics, yet have several mechanistic features in common. Both enzymes have ferriprotoporphyrin IX (protohemin; Fig. 8.3) as prosthetic groups (1 in peroxidase, 4 in catalase), and the chemistry of the reactions catalyzed by these enzymes very much includes what happens at this prosthetic group. The overall reactions catalyzed by the two enzymes are



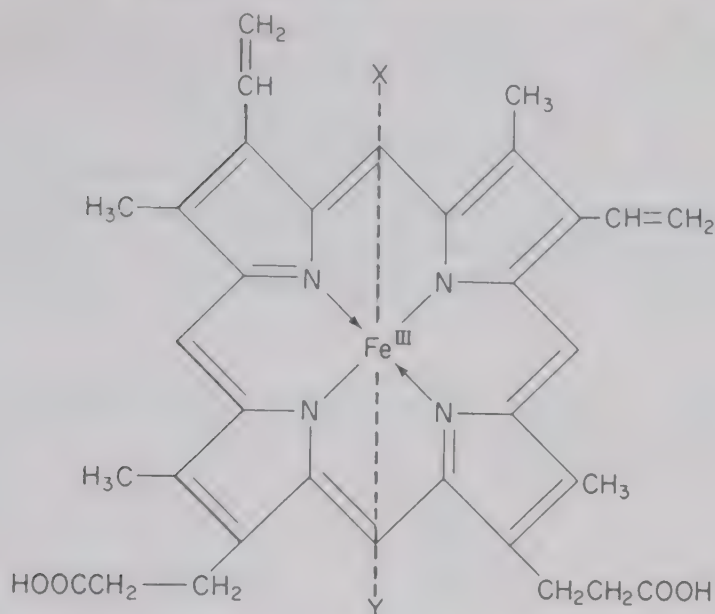


FIG. 8.3. Structure of ferriprotoporphyrin IX (ferric protohemin).

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where H_2O_2 is the acceptor molecule and AH_2 is the donor molecule. n is normally 2 or 4, as we shall see later. The first reaction is referred to as “catalatic” activity, the second reaction as “peroxidatic” activity.

Peroxidase

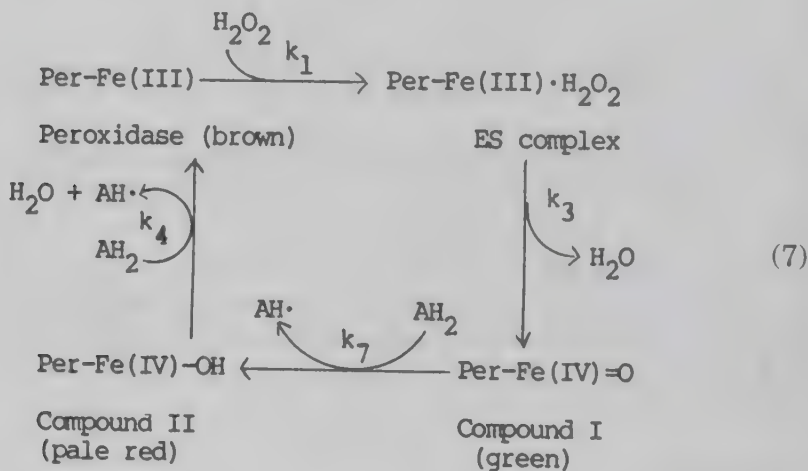
Peroxidase from horseradish is the best studied of the peroxidases and, unless indicated, all the data below are for this enzyme. It is unfortunate that more is not known about the peroxidases of the more commercially important plants, but the enzyme is in much higher concentration in horseradish than in other plants, making it easier to isolate from this source.

General Properties. The primary amino acid sequence of isoenzyme C (IIIb), the dominant isoenzyme in horseradish root, is known (Welinder 1979). The single polypeptide chain is 33,890 daltons. Attached is a single Fe protoporphyrin IX residue (550.5 daltons) and eight chains of carbohydrate (18% of total weight). The total weight of horseradish peroxidase-C is about 42,000. The eight chains of carbohydrate are attached via N-linkages to asparagine residues located at positions 13, 57, 158, 186, 198, 214, 250, and 268. The carbohy-

drate chains are all located on the surface of the protein. One tryptophan residue, possibly important in activity, is located at position 117. The three histidine residues are located at positions 40, 42, and 170.

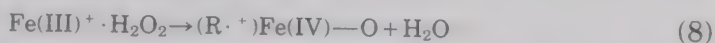
His-170, closest to the ferriprotoporphyrin IX and in a hydrophobic region of the amino acid sequence, probably forms the fifth coordinate position of the ferric ion. Four of the coordination bonds of the ferric ion are to the nitrogens of the pyrrole rings (Fig. 8.3). The sixth coordination position of the ferric ion is thought to be vacant in peroxidase (Spiro *et al.* 1979).

Mechanism. A general overall summary of the mechanism of peroxidase is shown by Eq. 7.

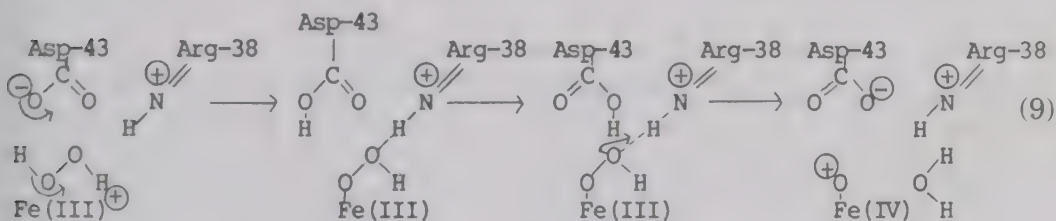


The resting peroxidase, in the Fe(III) state, is brown in color with a molar extinction coefficient at 403 nm of $1.08 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$. As reported above, the sixth coordination bond of the ferric ion does not appear to be filled with a water molecule, as originally thought (Spiro *et al.* 1979). Per-Fe(III) and H_2O_2 combine to give the enzyme-substrate complex with $k_1 = 9 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$ at pH 4.6 and 25°C . This is somewhat slower than a diffusion-controlled process. k_1 values for CH_3OOH and $\text{CH}_3\text{CH}_2\text{OOH}$ are 1.5×10^6 and $3.6 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$, respectively. The complex undergoes oxidation of the ferric ion to form Per-Fe(IV)=O with k_3 , a first-order rate constant, being faster than k_1 . The nature of Per-Fe(IV)=O is controversial. Based on proton nuclear magnetic resonance studies of compounds I and II of horseradish peroxidase, Morishima and Ogawa (1978) suggested the the π -cation radical on the heme ring can be ruled out as a source of oxidizing equivalent in compound I. However, Hanson *et al.* (1981) and others

(Dunford 1982) suggest that this does occur and that compound I formation should be written as (Eq. 8):



Formation of compound I appears to involve the side chains of Asp-43 and Arg-38. A proposed mechanism involving these two groups with the prosthetic group Fe(III) is shown in Eq. 9 (as adapted from Dunford and Araiso 1979 and Dunford 1982).



The conversion of Per-Fe(IV)=O (compound I) to Per-Fe(IV)—OH (compound II) occurs with a rate constant k_7 of 40–100 times faster than k_4 in the presence of a donor. If a second molecule of H₂O₂ serves as the donor, k_7 is 4.0 sec⁻¹; however, with *p*-hydroxydiphenyl it approaches 3–8 × 10⁹ M⁻¹ sec⁻¹ (second order and controlled by rate of combination of the donor). The step involves the protonation of compound I by removing a proton from the donor. The intermediate is pale red.

The conversion of Per-Fe(IV)—OH (compound II) to the resting enzyme Per-Fe(III) involves a second molecule of donor. If the donor is a molecule of H₂O₂, k_4 is 0.02 sec⁻¹; on the other hand, with *p*-hydroxydiphenyl, k_4 is 8 × 10⁷ M⁻¹ sec⁻¹, a second-order rate constant, indicating that enzyme–substrate complex formation is rate limiting. k_4 is considered to be the rate-limiting step for most donors.

In addition to peroxidatic activity, peroxidase also has catalatic, oxidatic, and hydroxylation activities, depending on the system. These reactions, in relation to the peroxidatic reaction, are shown in Fig. 8.4. The oxidatic and hydroxylation reactions are a result of formation of AH· in the reaction in the presence of O₂ (oxidatic) and monophenols (hydroxylation). These reactions are much slower than the peroxidatic reaction.

Catalase

General Properties. Catalase, like peroxidase, is found in animals, plants, and microorganisms. It has been purified from all three sources.

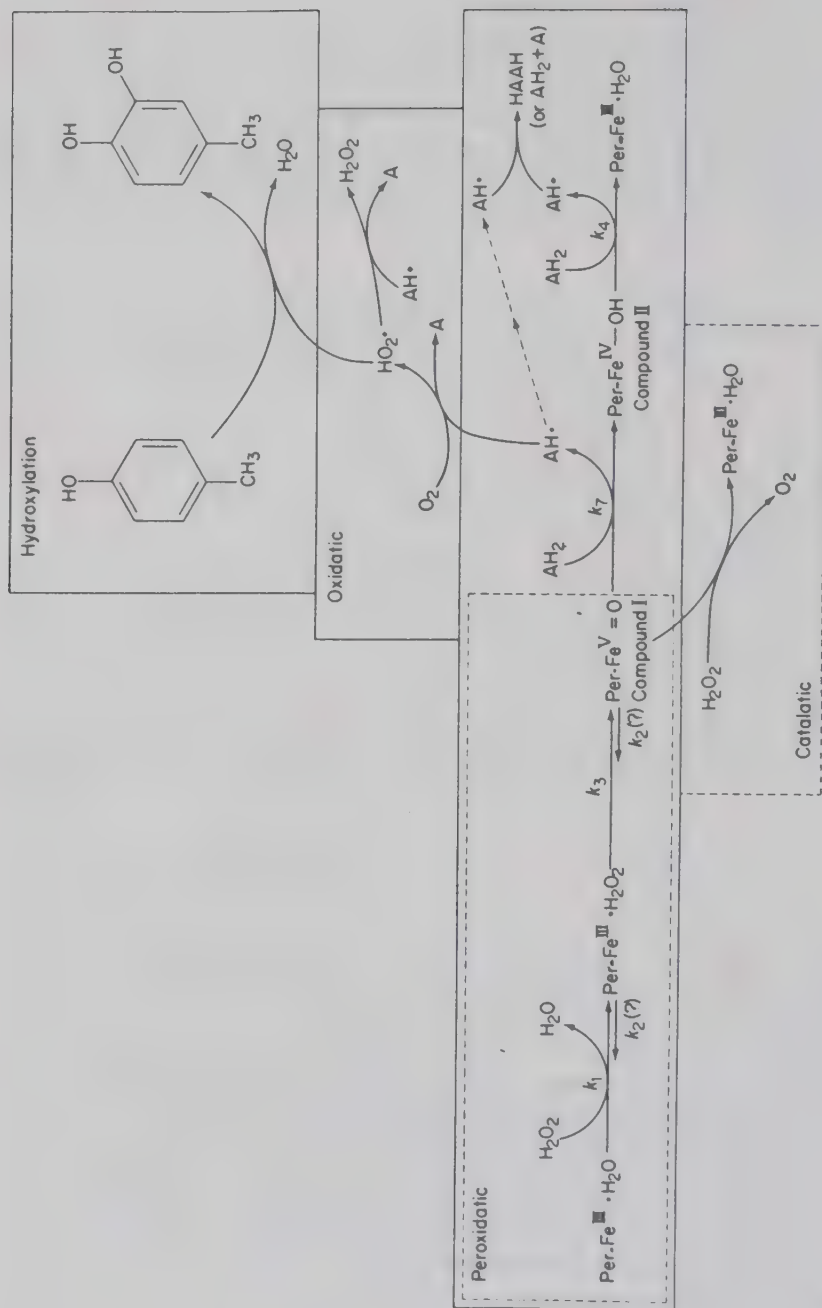


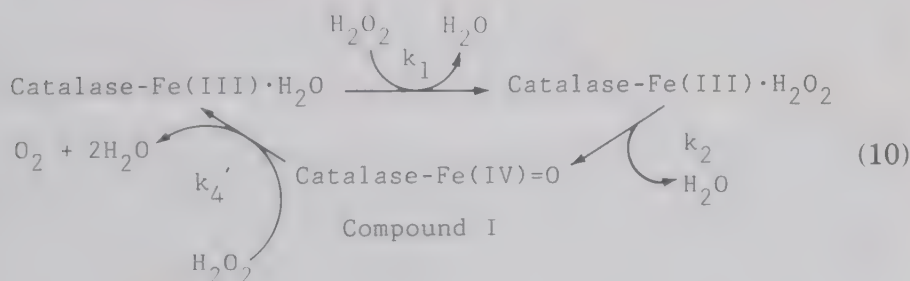
FIG. 8.4. Proposed scheme for mechanism of peroxidase in catalyzing the peroxidatic, catalatic, oxidative, and hydroxylation reactions.
 Reprinted from Whitaker (1972), p. 597, courtesy of Marcel Dekker, Inc.

Catalase, crystallized from beef liver in 1937 by Sumner and Dounce (1937), is the best studied enzyme. Most catalases have molecular weights around 240,000 and are composed of four identical subunits, each with a ferriprotoporphyrin IX prosthetic group. A recently isolated catalase from *N. crassa* has a molecular weight of 320,000 and differs in some other properties from most catalases (Jacob and Orme-Johnson 1979A, B).

Because of the subunit structure, the more complex structure, and the larger size, catalase is not as heat stable as peroxidase. Generally, it takes about half as much heat input to denature catalase as it does for peroxidase.

Mechanism. Catalase catalyzes two types of reactions, a catalatic and a peroxidatic reaction (Eqs. 5 and 6). Under normal reaction conditions, the catalatic reaction is greatly favored. For the peroxidatic reaction, very low concentrations of peroxide are used with hydrogen donors such as methanol, ethanol, and phenols.

Catalase can oxidize a number of hydrogen donors, as indicated in Fig. 8.5. The oxidation of hydrogen peroxide involves the reaction steps shown in more detail in Eq. 10.



k_1 values for H_2O_2 , CH_3OOH , and $\text{CH}_3\text{CH}_2\text{OOH}$ are 6×10^6 , 8.5×10^5 , and $2 \times 10^4 \text{ M}^{-1} \text{ sec}^{-1}$, respectively, at pH 7 and 25°C . There is more effect of the nature of the peroxide with catalase than with peroxidase. k_2 is faster than k_1 . k_4' for H_2O_2 is $\sim 1.8 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$, a second-order rate constant indicating that binding of the second H_2O_2 to catalase- Fe(IV)=O is the rate-limiting step. Note that compound II is not kinetically detectable when the donor molecule is H_2O_2 , nitrate, alcohols, or formic acid (Fig. 8.5). On the other hand, with ascorbate, ferrocyanide, and phenols, compound II is formed (Fig. 8.5).

When other donors are used, the rate constants are lower. In the case of methanol and ethanol, k_4' is $1 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$ for both and is $9 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ for formic acid. k_3 (Fig. 8.5) is 80 and $8 \text{ M}^{-1} \text{ sec}^{-1}$

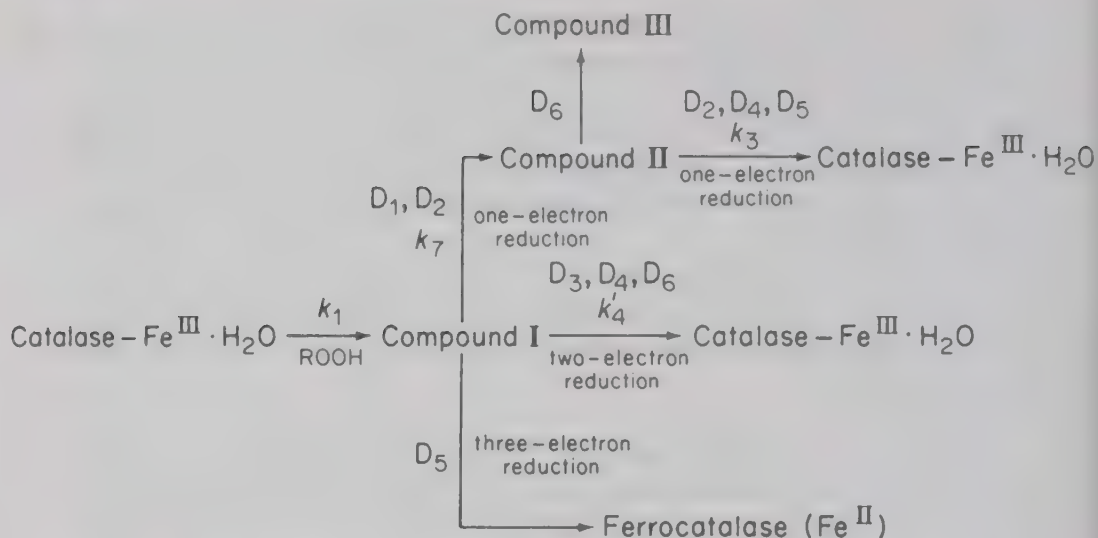


FIG. 8.5. The reactions of catalase with hydrogen donors. The Ds refer to the donors: D₁, ascorbate, ferrocyanide; D₂, phenols; D₃, alcohols, formic acid; D₄, nitrite; D₅, azide, hydroxylamine; D₆, hydrogen peroxide.

From Keilin and Nicholls (1958); Reprinted from Whitaker (1972), p. 601, courtesy of Marcel Dekker, Inc.

for pyrogallol and *p*-cresol, respectively, while k_7 is ~ 240 and $24 \text{ M}^{-1} \text{ sec}^{-1}$, respectively, for these compounds. These peroxidatic reactions are about four orders of magnitude slower than when catalyzed by peroxidase.

The catalatic activity of catalase is independent of pH over the range 3–9. Above pH 9, the enzyme dissociates into subunits, and below pH 3 the ferric protohemin group dissociates from the protein. The steps controlled by k_3 and k_7 (Fig. 8.5) are also pH independent when neutral donors are used. Since the rate-limiting step(s) with H₂O₂ as substrate is binding of the substrate to the enzyme (k_1 and k_4'), the reaction is almost temperature independent. The Q_{10} of the catalatic reaction above 25°C is 1.1 (Strother and Ackerman 1961).

XANTHINE OXIDASE, XANTHINE DEHYDROGENASE, AND ALDEHYDE OXIDASE

Introduction

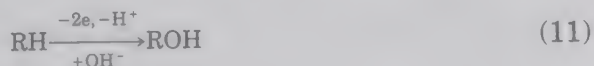
Xanthine oxidase is of interest to investigators in food science and technology primarily for two reasons. Xanthine oxidase, found in the liver of humans (and other animals), is involved in purine catabolism in which xanthine and hypoxanthine from the metabolism of nucleic

acids of the diet (particularly from meat) are converted to uric acid prior to excretion. Uric acid complexes with Ca^{2+} to form calcium urate of limited solubility; deposition of calcium urate in joints is associated with gout, the so-called rich man's disease. There is little evidence that this is a necessary role of xanthine oxidase in humans; in fact, there is more evidence to indicate that humans would be better off without the enzyme-catalyzed reaction. The presence of active xanthine oxidase in pasteurized homogenized milk has led to the unwarranted conclusion that this xanthine oxidase will be absorbed through the mucosa of the small intestine, leading to increased incidents of heart disease (Oster and Ross 1983). Clifford and his co-workers, through carefully performed experiments (Clifford *et al.* 1983), have shown that these conclusions of Oster and Ross are without substance.

Xanthine oxidase (xanthine:oxygen oxidoreductase; EC 1.2.3.2) was discovered almost simultaneously by two different groups shortly after 1900. An enzyme in mammalian tissues was shown to catalyze the oxidation of xanthine and hypoxanthine to uric acid. At almost the same time, an enzyme was found in bovine milk which oxidized aldehydes to carboxylic acids. In 1935 it was shown that the two enzymes are the same.

General Properties

Xanthine oxidase belongs to the molybdenum hydroxylase group of enzymes, along with the less well-investigated enzymes xanthine dehydrogenase (EC 1.2.1.37) and aldehyde oxidase (EC 1.2.3.1). All three of these enzymes have broad specificities which overlap to a large extent. All contain FAD, molybdenum, and iron-sulfur centers (see below). The overall general reaction catalyzed by this group of enzymes (Eq. 11) is the oxidation of xanthine, hypoxanthine, and aldehydes by the oxygen from water, as shown by H_2^{18}O studies.



The best studied xanthine oxidase is that from bovine milk where the concentration is usually around 50 mg enzyme per liter of milk. Unless otherwise indicated, all properties described here are for xanthine oxidase from that source. The average molecular weight (by several methods and from several laboratories) is 283,000 (Bray 1976). It is composed of two identical subunits of ~150,000 molecular weight, each containing 1 Mo, 1 FAD, 4 Fe, and 4 acid-labile S (as $2 \text{Fe}_2\text{S}_2$

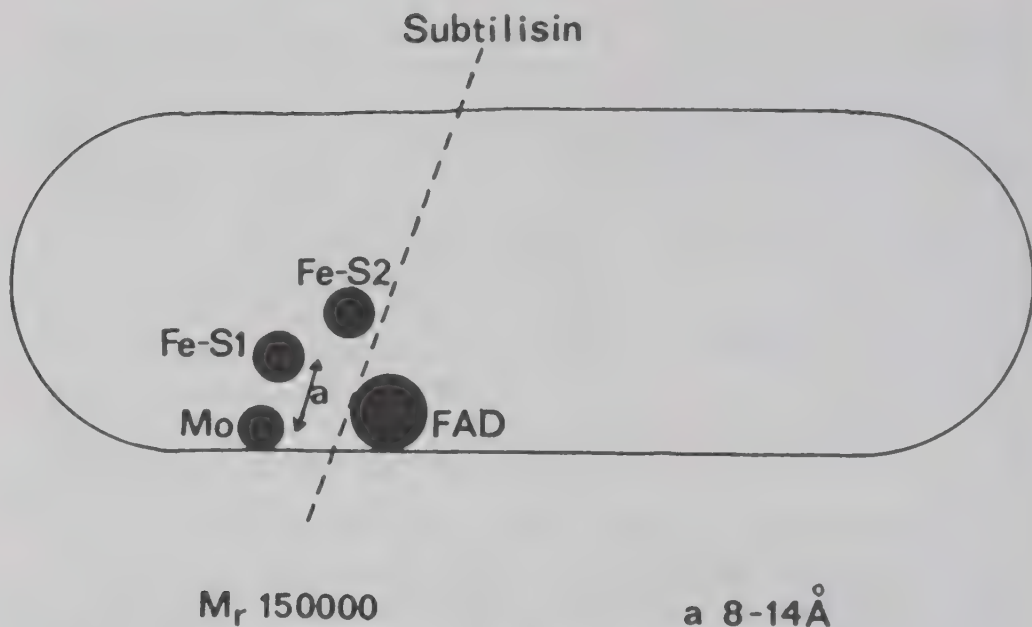


FIG. 8.6. Schematic representation of the approximate size and location of the four redox-active centers in one subunit of xanthine oxidase. The distance between Mo and Fe-S₁ is known to be 8–14 Å; the other distances are more arbitrary. Proteolysis with subtilisin produces two fragments, one containing the FAD binding part, the other the Mo, Fe-S₁, and Fe-S₂ binding part.

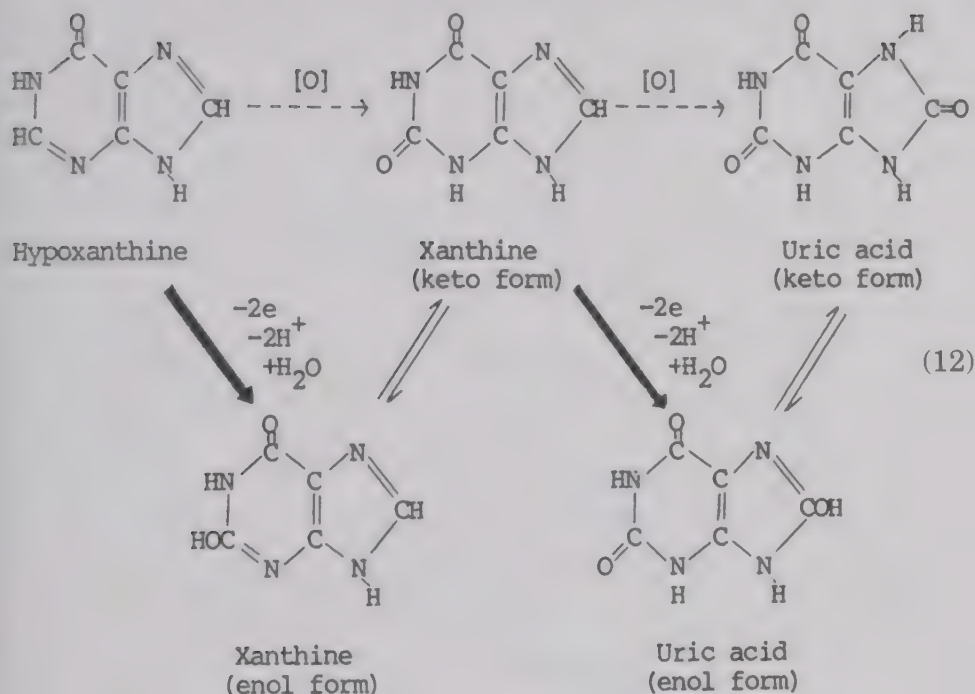
Reprinted by permission of the publisher from Bray (1982), p. 776, Elsevier Science Publishing Co., Inc.

centers). The enzyme has a pH optimum at 8.3 and an isoelectric point of 5.3–5.4. The demolybdo enzyme is also present in milk, presumably being about 50% of the total enzyme. It can be selectively removed by denaturation with salicylate (Hart *et al.* 1970).

An approximate schematic arrangement of the four cofactors (four redox-active centers) on each subunit is shown in Fig. 8.6. The exact location of the four redox-active centers is not known. Mo and FAD are thought to be close to the surface of the molecule, but the locations of Fe-S₁ and Fe-S₂ are not known. The Mo and Fe-S₁ centers are 8–14 Å apart. Since electron transfer among the centers occurs rapidly (and most likely without contact with the solvent), all the redox-active centers are likely to be within 20 Å of each other.

Reaction Pathway

The overall xanthine oxidase-catalyzed oxidation of xanthine and hypoxanthine to uric acid is shown in Eq. 12.



Hypoxanthine oxidation to xanthine requires the removal of two electrons and two hydrogen ions and the addition of one H_2O (heavy arrow in Eq. 12), as does the oxidation of xanthine to uric acid (heavy arrow, Eq. 12).

As indicated above, xanthine oxidase has broad specificity, being able to oxidize several purines to uric acid as well as both aliphatic and aromatic aldehydes to the corresponding carboxylic acids (Table 8.3).

TABLE 8.3. SUBSTRATE SPECIFICITY OF BOVINE MILK XANTHINE OXIDASE

Substrate	Relative rate ^a	Substrate	Relative rate ^a
Xanthine	140	<i>p</i> -Hydroxybenzaldehyde	1.3
Hypoxanthine	100	Benzaldehyde	0.8
8-Hydroxypurine	6	Cinnamaldehyde	0.41
6-Amino-2-hydroxypurine	4.5	<i>p</i> -Phthalaldehyde	0.013
6-Amino-8-hydroxypurine	7	Vanillin	0.01
Adenine	6	Acetaldehyde	0.72
2,8-Dihydroxypurine	0.6	Glyceraldehyde	0.014
		Decanaldehyde	0.0080
		<i>n</i> -Butyraldehyde	0.0060
		Octaldehyde	0.0002

Source: Booth (1938).

^a Relative to hypoxanthine = 100.

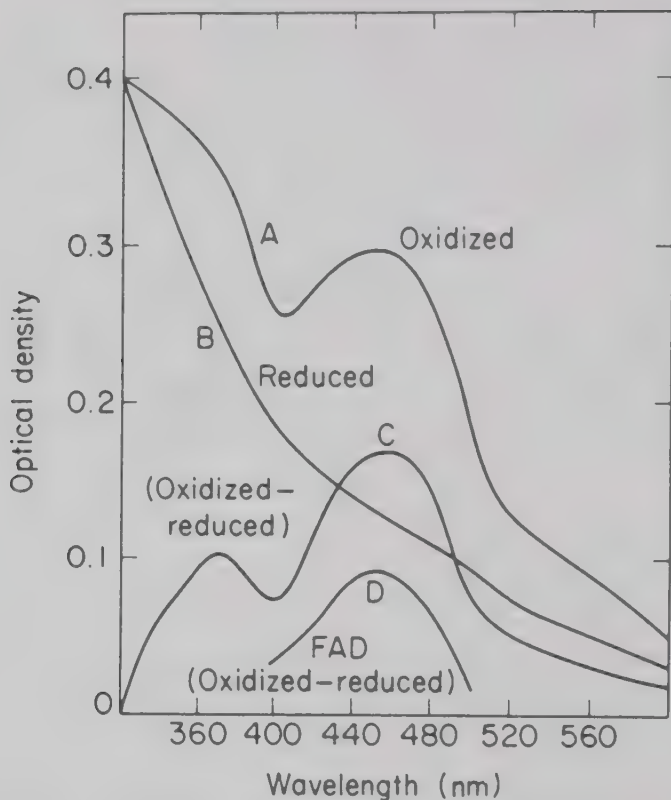


FIG. 8.7. Absorbance spectrum of xanthine oxidase. A, Oxidized form; B, after complete reduction with xanthine under anaerobic conditions; C, the difference spectrum between oxidized and reduced enzyme; and D, the difference spectrum between FAD and FADH₂ at the same concentration.

From Morell (1952); Reprinted from Whitaker (1972), p. 586, courtesy of Marcel Dekker, Inc.

Oxidation of substrates by xanthine oxidase is done in two half reactions. The first half reaction, involving the removal of 2 H⁺ from the substrate along with 2 e⁻, can be performed under anaerobic conditions in the absence or presence of electron acceptors other than O₂ (examples of acceptors are Methylene Blue, 2,6-dichlorophenol-indophenol, triphenyltetrazolium chloride, phenazine methosulfate, cytochrome *c*, and ferricyanide). When performed in the absence of acceptor, there is a decrease in absorbance at 450 nm due to reduction of FAD (Fig. 8.7), increase in absorbance at 610 nm due to semiquinone formation (EFAD → EFADH[•]; not shown), and changes in EPR signals (Fig. 8.8) involving the formation of EFADH[•], Mo(V) from Mo(VI), and Fe(III) from Fe(II) (Bray *et al.* 1964).

The pathway of electron flow through the four redox-active centers has been the subject of intense investigation. Based on the time se-

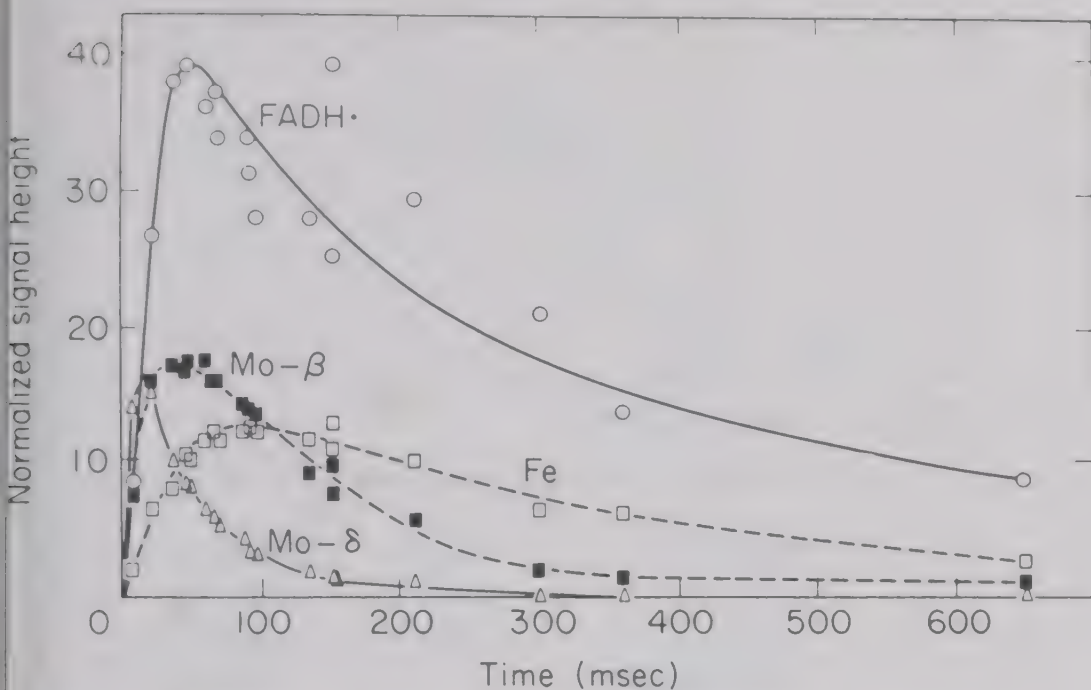
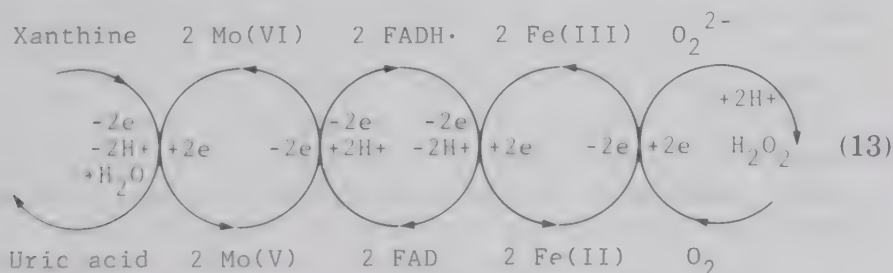


FIG. 8.8. Electron paramagnetic resonance signals generated by xanthine oxidase in a "single turnover" experiment with xanthine as substrate.

From Bray *et al.* (1964); Reprinted from Whitaker (1972), p. 589, courtesy of Marcel Dekker, Inc.

quence of rise and fall in ESR signals generated by xanthine oxidase in a "single turnover" experiment with xanthine as substrate, Bray *et al.* (1964) concluded that the order of transfer of electrons is that indicated in Eq. 13.



More recently, based primarily on lack of stoichiometric reduction of the four redox-active centers (Olson *et al.* 1974A) and on the redox potentials of all four redox-active centers being independent of each other, Bray (1982) has concluded that the electrons, initially accepted from the substrate by Mo(VI), are distributed among the four redox-

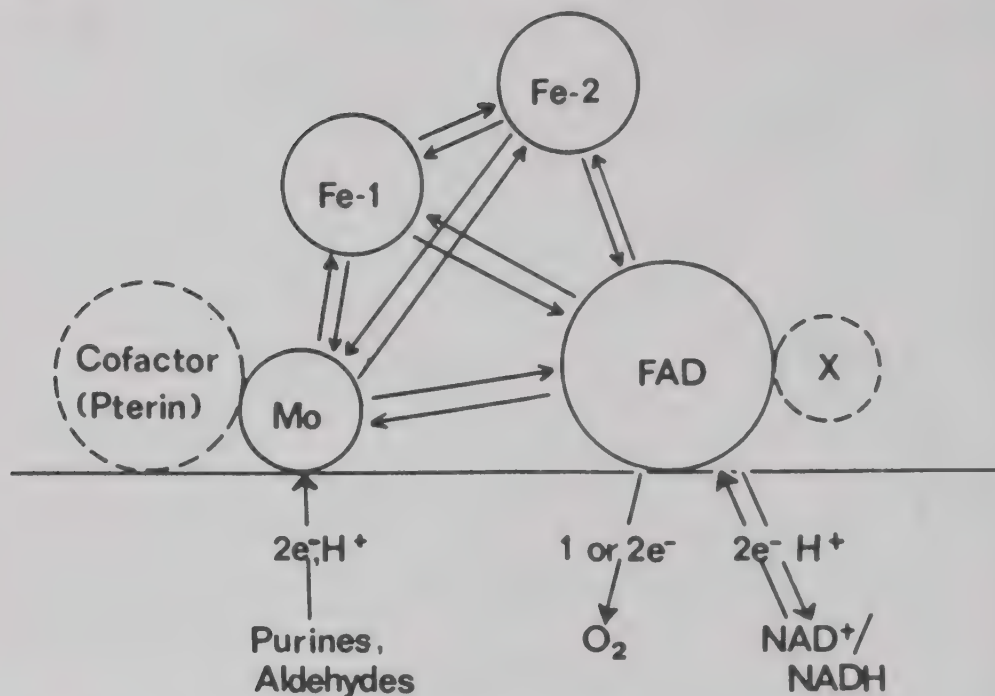
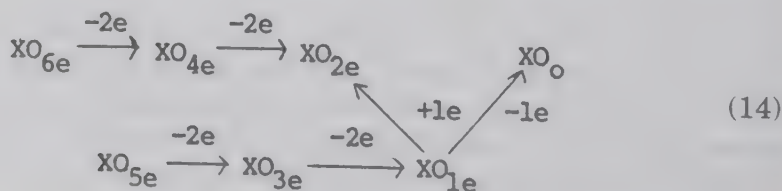


FIG. 8.9. Xanthine oxidase reaction with substrates (purines and aldehydes) and intramolecular electron transfer reactions. X could be a NAD⁺ molecule as is present in xanthine dehydrogenase (for example). The cofactor pterin may be important in the binding of Mo into the demolybdo enzyme.

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active centers as predicted from the redox potentials of each site (Fig. 8.9). Intramolecular electron transfer among the four redox-active centers does not appear to be rate determining, since k is $> 100 \text{ sec}^{-1}$ (Olson *et al.* 1974B) while the turnover number for xanthine is extrapolated to be 16.2 sec^{-1} at pH 8.2 and 23.5°C (Massey *et al.* 1969).

A maximum of six electrons can be taken up by each enzyme half molecule in full reduction either by one-electron (Olson *et al.* 1974A) or two-electron (Barber *et al.* 1977) reducing agents. Olson *et al.* (1974A) proposed that the one- and two-electron oxidation steps primarily occurred in the order shown in Eq. 14.



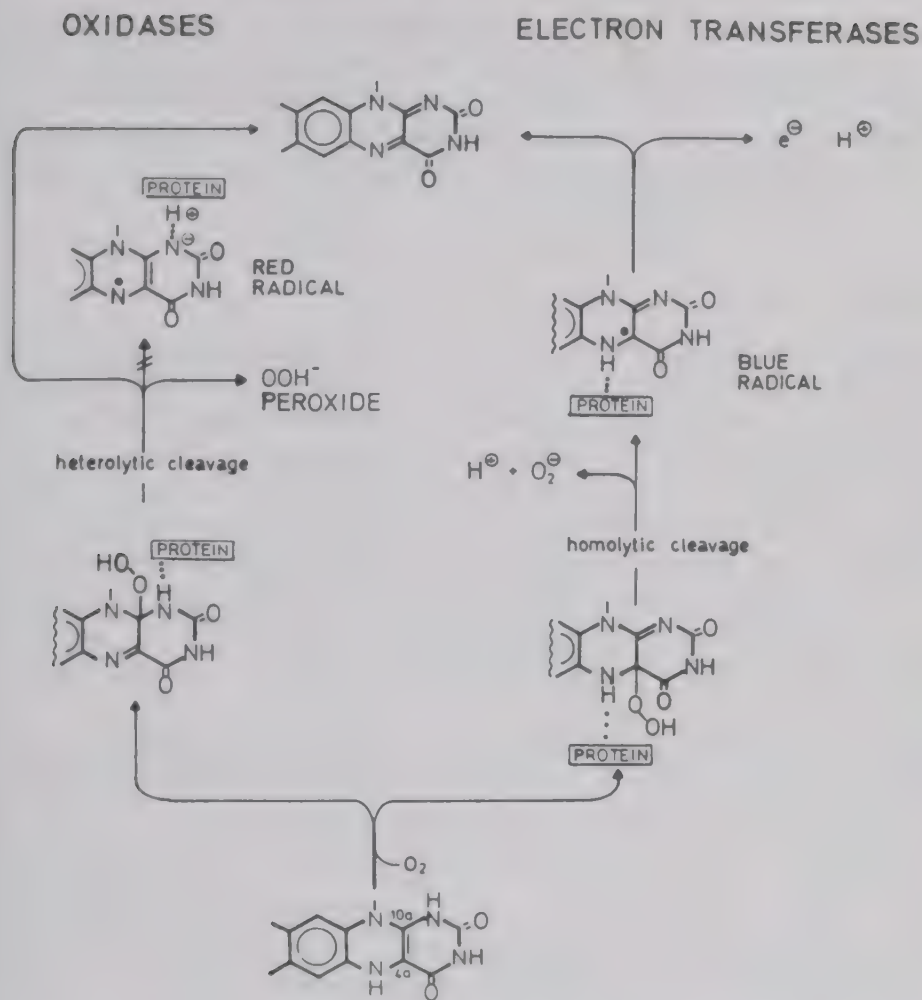


FIG. 8.10. Possible pathways first for two-electron oxidations ($6 \rightarrow 4 \rightarrow 2$) in xanthine oxidase (oxidases side) followed by one-electron oxidations ($2 \rightarrow 1 \rightarrow 0$).

From Hemmerich and Massey (1981), as depicted in Malmström (1982); Reproduced, with permission, from the *Annual Review of Biochemistry*, Vol. 51. © 1982 by Annual Reviews Inc.

The differently reduced forms of xanthine oxidase are chemically and spectrophotometrically distinct. The one- and two-electron reduced forms are the only ones that react with O_2 to give superoxide ($O_2^{\cdot -}$). The more extensively reduced forms of the enzyme give H_2O_2 only.

Figure 8.10 shows a suggested reaction of flavins with O_2 when xanthine oxidase acts as an oxidase (left side) and as an electron transferase (Hemmerich and Massey 1981). The two-electron oxidation steps, from $6 \rightarrow 4 \rightarrow 2$, give H_2O_2 . At the two-electron reduced stage, however, xanthine oxidase switches from an oxidase to an electron

transferase, involving one-electron transfer processes ($2 \rightarrow 1 \rightarrow 0$). These last two steps must generate $O_2^- \cdot$. In the normal catalytic turnover of xanthine oxidase, where there is plenty of reducing substrate, $O_2^- \cdot$ formation would be minimized. Therefore, it is felt xanthine oxidase produces H_2O_2 directly, as shown in Fig. 8.10, rather than via an $O_2^- \cdot$, as was previously thought.

Kinetics

As might be expected from the discussions above showing that the oxidation of substrate occurs in two half reactions, steady-state kinetics show that the reaction follows a modified ping-pong bi-bi mechanism (Figs. 8.11 and 8.12; Coughlan and Rajagopalan 1980). The system is probably more complex than a classical ping-pong bi-bi mechanism because up to three substrate molecules can be bound and oxidized as the enzyme cycles from the fully oxidized (XO_0) to the fully reduced (XO_{6e}) forms (Eq. 14). The Cleland diagram for the sequence of events observed kinetically is shown in Eq. 15, where E and F are the oxidized and reduced forms of the enzymes, respectively.

Transient kinetics of xanthine oxidase measured spectrophotomet-

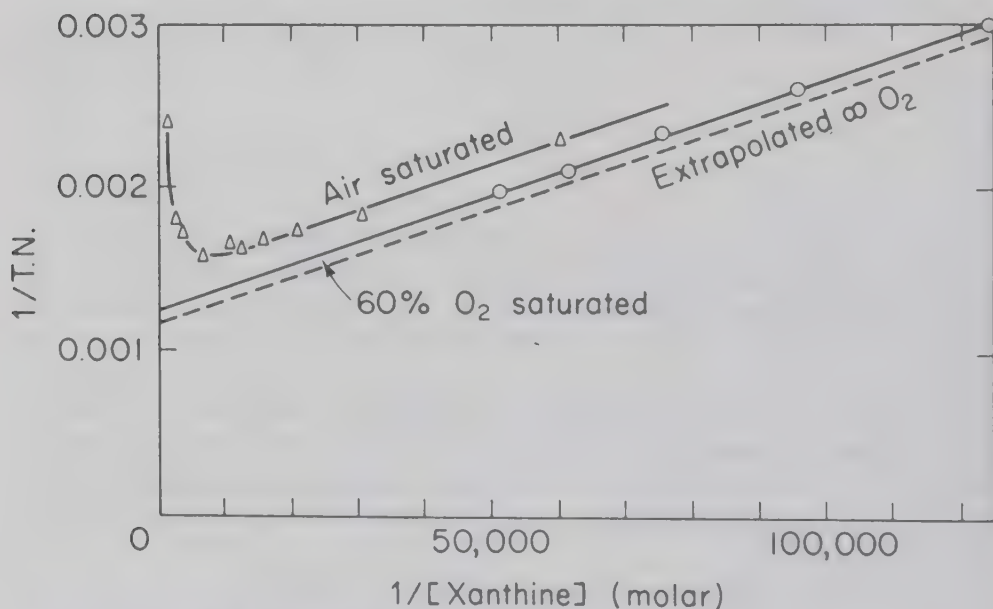


FIG. 8.11. Effect of xanthine concentration on the rate of xanthine oxidation at different concentrations of O_2 . T.N. is the turnover number measured at pH 8.3 and $25^\circ C$.

From Massey et al. (1969); Reprinted from Whitaker (1972), p. 587, courtesy of Marcel Dekker, Inc.

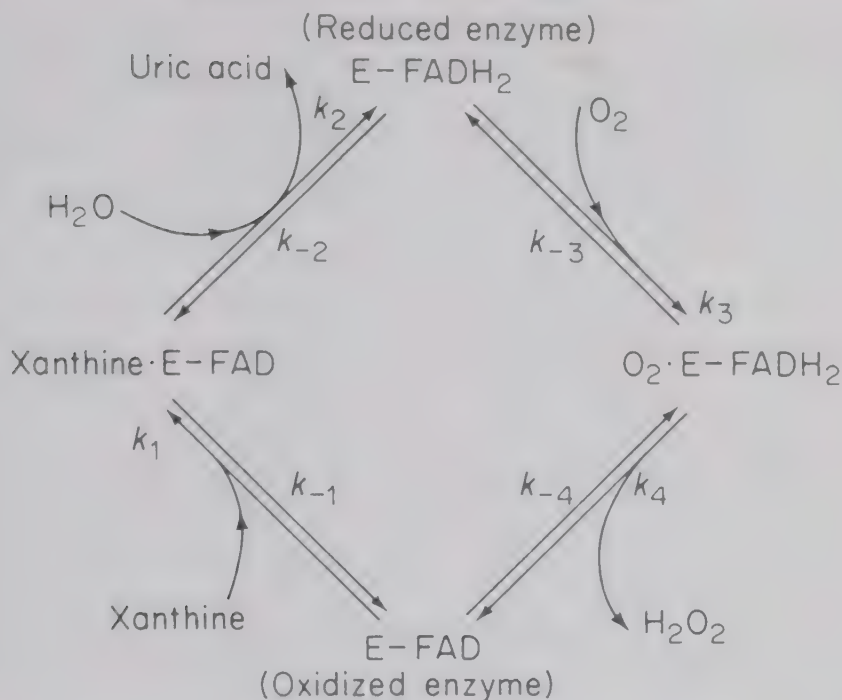
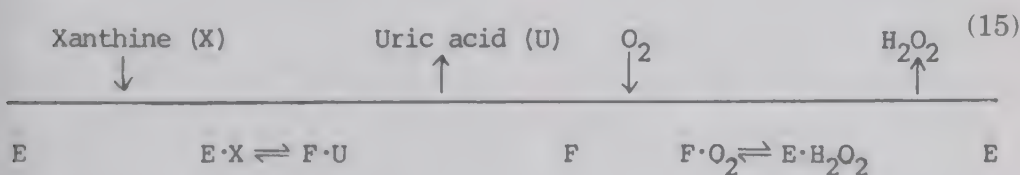


FIG. 8.12. Mechanism of action of xanthine oxidase as determined kinetically. From Gutfreund and Massey (1959); Reprinted from Whitaker (1972), p. 588, courtesy of Marcel Dekker, Inc.



rically are in accord with the reaction pathway shown in Eq. 13. As shown in Fig. 8.12, xanthine is bound to the oxidized enzyme EFAD to form the Michaelis complex with k_1 of $\sim 5.0 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$. In the second step, uric acid is formed with a rate constant k_2 of 10.5 sec^{-1} . The reduced enzyme EFADH₂ combines with O₂ with a rate constant k_3 of $> 4.0 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ and is oxidized to EFAD and formation of H₂O₂ with a rate constant k_4 of 21.5 sec^{-1} . Therefore, the oxidation of xanthine and the reduction of O₂ are rate-determining steps in the overall reaction.

Mechanism

Based on many years of work, both from kinetic and from chemical studies, the best mechanism appears to be that shown in Fig. 8.13

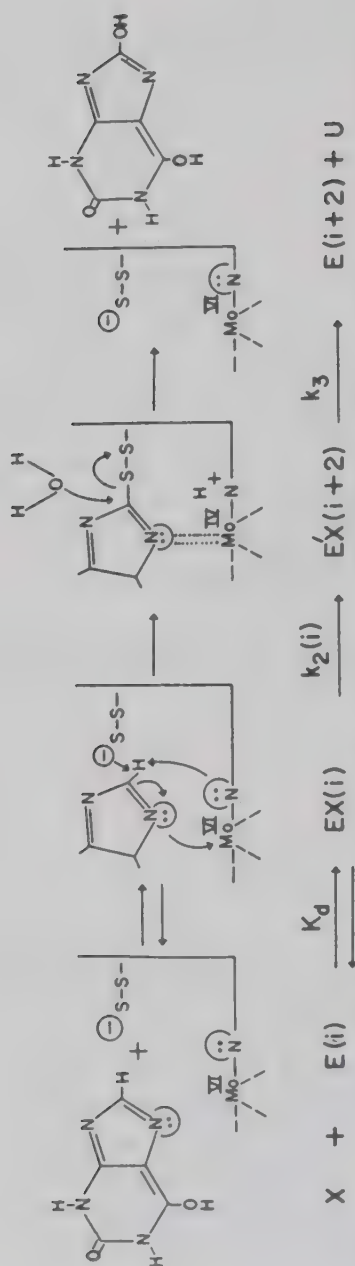


FIG. 8.13. Kinetic and chemical schemes representing the reduction of xanthine oxidase by xanthine, as proposed by Olson *et al.* (1974a). $EX(i)$ is a Michaelis complex and $E'X(i+2)$ an intermediate in which the xanthine residue has become covalently bound and two electrons have been transferred to the enzyme and reside mainly on the molybdenum. In $E(i+2)$, the reaction has been completed and the product has dissociated. The two electrons of the reduced enzyme are now localized mainly on the flavin and Fe/S centers.

Reprinted from Olson *et al.* (1974a; Bray (1975).

(Olson *et al.* 1974A). The proposed mechanism of substrate hydroxylation (e.g., xanthine) involves the following sequential steps: (1) coordination of substrate to molybdenum (Michaelis complex); (2) transfer of a proton from the substrate to an accepting group (most likely a terminal sulfur ligand) and the two electrons to Mo(VI); (3) the substrate carbonium ion is stabilized by reaction with a nucleophile (terminal oxygen ligand of molybdenum; $\text{S}=\text{Mo}-\text{O}-\text{C}=\text{O}$). Product (e.g., uric acid) release takes place by a hydroxide ion attack (from H_2O) and cleavage of the molybdenum oxygen bond, to release the hydroxylated product.

While xanthine oxidase, xanthine dehydrogenase, and aldehyde oxidase have many properties in common [molecular weights near 300,000, two identical subunits, inhibition by arsenite (Barber and Siegel 1983), 1 Mo, 1 FAD, 4 Fe, 4 S (as 2 Fe_2S_2 centers)], there are important differences (Bray 1982; Barber *et al.* 1982). Xanthine oxidase utilizes O_2 effectively as a reducing agent, but not NAD^+ , while xanthine dehydrogenase uses NAD^+ much more effectively than it does O_2 . It is thought that those molybdenum hydroxylases able to use NAD^+ contain a binding site for NAD^+ (possibly X in Fig. 8.9), while the others do not. A second difference is in the redox potentials of $\text{EFADH}\cdot/\text{EFADH}_2$. The $\text{EFAD}/\text{EFADH}\cdot$ potentials are about -350 to -360 mV for both milk xanthine oxidase and turkey liver xanthine dehydrogenase, but the $\text{EFADH}\cdot/\text{EFADH}_2$ potential is -236 mV in the milk enzyme, while it is -326 mV for the turkey enzyme. Therefore, the prevalence of $\text{EFADH}\cdot$ over EFADH_2 (needed to react with O_2) probably explains why the xanthine dehydrogenase does not react readily with O_2 . A third difference among the three enzymes is the ability to form superoxide (Nakamura and Yamazaki 1969). This may be related to the ability to form Mo_{5e} and Mo_{4e} vs more extensively oxidized forms of the enzyme (see Eq. 14). Barber *et al.* (1982) have pointed out differences in properties of the individual Fe-S centers and the magnetic interactions between aldehyde oxidase and xanthine oxidase/xanthine dehydrogenase.

In conclusion, it is interesting to note similarities between xanthine oxidase and other molybdenum hydroxylases and nitrate reductase. Not only do the enzymes contain similar cofactors, but the molybdenum of denatured xanthine oxidase activates nitrate reductase while free molybdenum does not (Bray 1976). This suggests that there is a common molybdenum cofactor ($\text{Mo} + ?$) in these enzymes (Nason *et al.* 1971).

GLUCOSE OXIDASE

Introduction

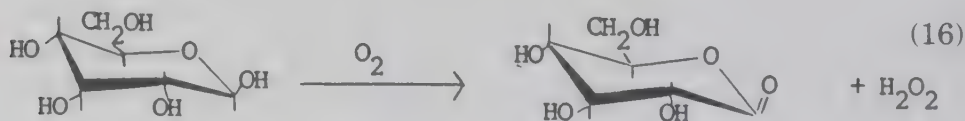
Glucose oxidase (β -D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) is well known in food science and technology, primarily due to three uses. Coupled with catalase, it is used (or has been used) extensively to remove O_2 from the head space of wines and catsups, packaged dry soups, etc. to prevent oxidation of pigments, lipids, etc. It is also used in removal of glucose from dried egg whites in order to prevent browning (Maillard reaction involving glucose and the ϵ -amino group of lysyl residues of proteins). Glucose oxidase is of major use for the analytical determination of glucose in blood and urine, in foods, and in other biological systems.

Glucose oxidase was discovered in 1928 by Muller (Muller 1928) in *Aspergillus niger* and in *Penicillium glaucum*. It has also been demonstrated in other fungi, including *Aspergillus oryzae*, *Penicillium anagaskiense*, and *Penicillium notatum*. It is not found in higher plants or in animals.

General Properties

The glucose oxidases from the different fungi are similar in that they all have molecular weights near 160,000–180,000, contain 2 mol of FAD per mol of enzyme, and have isoelectric points near pH 4.2. The best studied glucose oxidase is that from *A. niger* which has a molecular weight of 186,000 and is a dimer.

The reaction catalyzed by glucose oxidase is the oxidation of β -D-glucose (best substrate) by molecular oxygen to δ -D-gluconolactone and hydrogen peroxide (Eq. 16).



The δ -gluconolactone is slowly hydrolyzed nonenzymatically to δ -gluconic acid. Other hydrogen acceptors such as thionine, Methylene Blue, and quinones can replace O_2 . However, the end product is not H_2O_2 . Glucose is also oxidized to δ -D-gluconolactone by glucose dehydrogenase (β -D-glucose:NAD(P)⁺ 1-oxidoreductase; EC 1.1.1.47) found in bovine liver. Glucose dehydrogenase requires NAD⁺ as cofactor, performs the reaction anaerobically as well as aerobically, and does not produce H_2O_2 as a product. Glucose oxidase is similar, mechani-

TABLE 8.4. SUBSTRATE SPECIFICITY OF GLUCOSE OXIDASE

Position modified on glucose	Compound	Change from β -D-glucose	Relative rate
1	β -D-Glucose ^a	None, reference compound	100
	α -D-Glucose ^a	Configuration of OH at C-1	0.64
	1,5-Anhydro- β -D-glucitol ^b	Replacement of C-1 OH with H	0
2	2-Deoxy- β -D-glucose ^c	Replacement of C-2 OH with H	3.3
	D-Mannose ^a	Configuration of OH at C-2	0.98
	2-O-Methyl- β -D-glucose ^a	Substitution of hydrogen of C-2 OH with methyl	0
3	3-Deoxy- β -D-glucose ^b	Replacement of C-3 OH with H	1
4	β -D-Galactose ^b	Configuration of OH at C-4	0.5
	4-Deoxy- β -D-glucose ^b	Replacement of C-4 OH with H	2
5	5-Deoxy- β -D-glucose ^b	Replacement of C-5 OH with H (no pyranose ring can form)	0.05
	L- β -D-glucose ^b	Configuration at C-5	0
6	6-Deoxy- β -D-glucose ^b	Replacement of C-6 OH with H	10
	Xylose ^a	Replacement of C-6 CH ₂ OH group with H	0.98

^a Keilin and Hartree (1948).^b Pazur and Kleppe (1964).^c Bright and Appleby (1969).

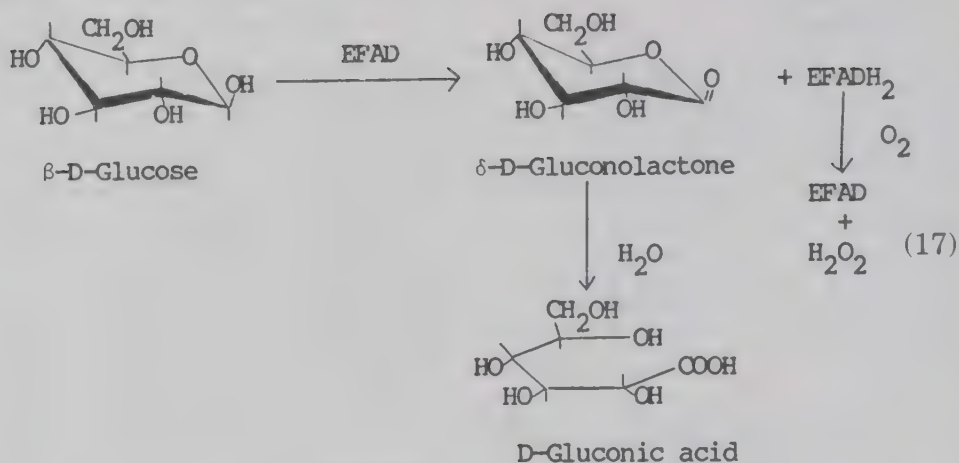
cally, to some 20 other well-described flavoenzymes (see EC 1.1.3.1, -4, -5, -12, -13, and -15; EC 1.2.3.3, -4, -5; EC 1.3.3.1; EC 1.4.3.1, -2, -3, -4, -5, and -9; EC 1.5.3.2, -5 and -6; EC 1.6.99.1; and EC 1.7.3.1; Bright and Porter 1976). The best characterized of these enzymes, in addition to glucose oxidase, are L-amino oxidase (EC 1.4.3.2), D-amino acid oxidase (EC 1.4.3.3), monoamine oxidase (EC 1.4.3.4), and Old Yellow enzyme (EC 1.6.99.1).

Glucose oxidase is much more specific for β -D-glucose than for α -D-glucose or other monosaccharides, making it ideal not only for quantitatively determining glucose in solutions, but also to distinguish between α - and β -D-glucoses. The relative rates on several monosaccharides are shown in Table 8.4. From the data given, one can determine the effect on the relative rates by modification at every position of glucose. As shown, glucose oxidase "feels" every part of the molecule in forming the enzyme-substrate complex and/or in catalysis. Such complete data on substrate specificity are rarely available for enzymes.

Kinetics and Mechanism

Glucose oxidase performs the overall reaction shown in Eq. 17 in two parts (two half reactions), as shown by the following kinetic anal-

yses of the system:

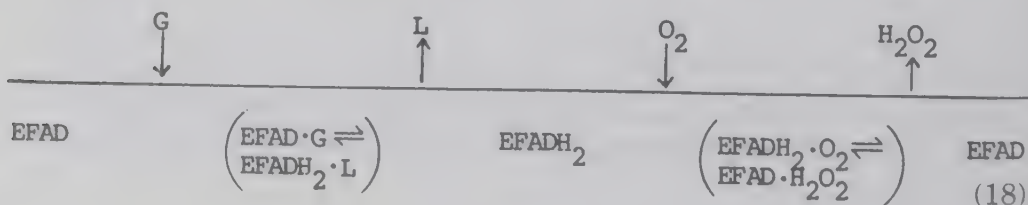


1. When glucose and glucose oxidase are combined under anaerobic conditions, δ -D-gluconolactone (and with hydrolysis, D-gluconic acid) is formed in stoichiometric amounts with FAD content of the enzyme. There is a disappearance of the absorbance maxima at 377 and 455 nm due to FAD reduction to FADH₂.

2. On admittance of O₂ to the anaerobic reaction in 1, the absorbance maxima at 377 and 455 nm reappear, H₂O₂ is formed, and glucose oxidase cycles repetitively in the system.

3. Plots of 1/rate vs 1/[O₂] (Lineweaver-Burk plots) at several different glucose concentrations give a series of parallel lines (Fig. 8.14). Plots of 1/rate vs 1/[glucose] at several different O₂ concentrations also give a series of parallel lines (Gibson *et al.* 1964; replot of data in Fig. 8.14). These data indicate not only a dependence of rate on both O₂ and glucose concentrations, they also indicate that the mechanism of the reaction catalyzed by glucose oxidase is a ping-pong bi-bi mechanism. Diagrammed by the Cleland method (Cleland 1963A, B, C), the reaction catalyzed by glucose oxidase is shown in Eq. 18, where G and L are β -D-glucose and δ -D-gluconolactone, respectively.

As shown by Eq. 17, the oxidized form of the enzyme, EFAD, functions as a dehydrogenase to remove two hydrogens from the C-1 position of β -D-glucose to form the reduced enzyme, EFADH₂, and δ -D-gluconolactone. The reduced enzyme, EFADH₂, is reoxidized to EFAD



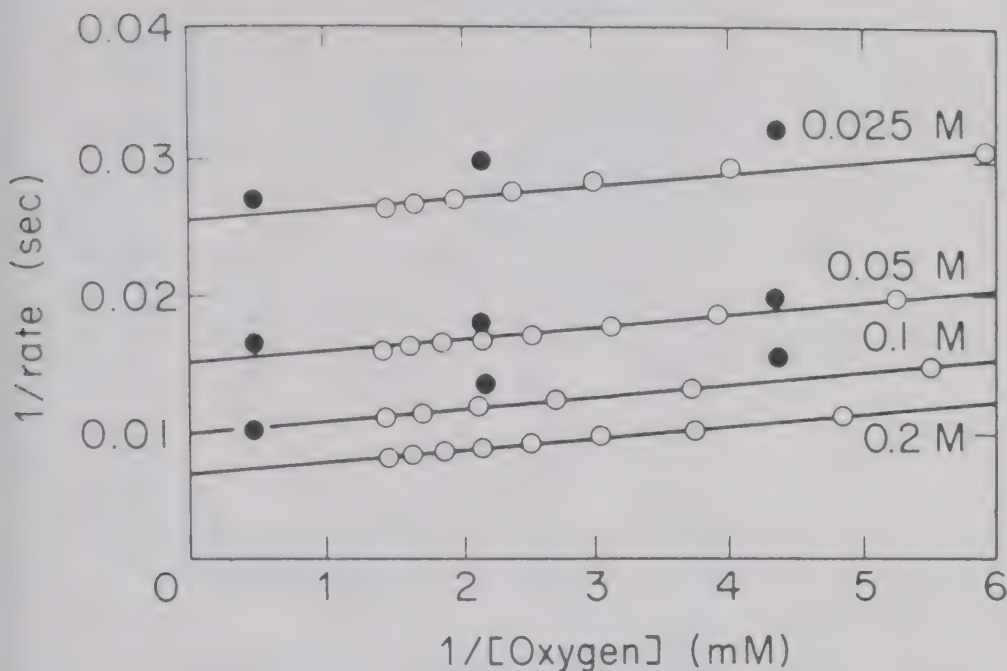


FIG. 8.14. Rate of oxidation of glucose by glucose oxidase at various concentrations of O_2 and of glucose. The glucose concentrations are given on the graph. (●), Manometric experiments; (○), stopped flow spectrophotometric experiments; 0° , pH 5.6.

From Gibson et al. (1964); Reprinted from Whitaker (1972), p. 567, courtesy of Marcel Dekker, Inc.

by O_2 , forming also H_2O_2 . δ -D-Gluconolactone is hydrolyzed to D-gluconic acid nonenzymatically.

The complete pathway of the glucose oxidase-catalyzed oxidation of β -D-glucose has been elucidated by using the relatively poor substrates, D-mannose and 2-deoxy-D-glucose, along with the best substrate, β -D-glucose (Bright and Appleby 1969). Fortunately, there is a different rate-determining step for each substrate (Table 8.5). Because of the different rate-determining steps, each substrate gives a different pH activity profile, allowing one to determine the prototropic forms of the free enzyme and enzyme-substrate species which are active (Bright and Appleby 1969; Fig. 8.15; Table 8.5).

By investigating the rates of the two half reactions (Eq. 17) spectrophotometrically as a function of pH using steady-state and transient kinetics, Bright and Appleby (1969) elucidated all the rate constants and their pH dependencies for the oxidation of β -D-glucose, D-mannose, and 2-deoxy- β -D-glucose by glucose oxidase. As shown in Table 8.5, the rate-determining step for the oxidation of β -D-glucose is the dissociation of H_2O_2 from $\text{EFAD} \cdot \text{H}_2\text{O}_2$ (step controlled by k_6). The H_2O_2 can only be released from the prototropic form

TABLE 8.5. KINETIC CONSTANTS AND pK_a VALUES FOR PROTOTROPIC GROUPS OF GLUCOSE OXIDASE-SUBSTRATE SPECIES INVOLVED IN OXIDATION OF THREE MONOSACCHARIDES

Substrate	k_1^a ($M^{-1} \text{ sec}^{-1}$)	k_2 (sec^{-1})	k_4 ($M^{-1} \text{ sec}^{-1}$)	k_6 (sec^{-1})	pK_1^a	pK_4	pK_5	pK_5'	$V_{\max}^b$ ($M \text{ sec}^{-1}$)	K_m^b (sugar) (M)	K_m^b (O_2) (mM)
β -D-Glucose	12,600	3400	2.5×10^6	1200	5.00	6.90	4.10	7.40	1150	0.11	0.48
2-Deoxy- β -D-glucose	1,400	40			5.35				50	0.04	0.03
D-Mannose	15				5.00				22		

Source: Bright and Appleby (1969).

^a See Eq. 19 for meaning of rate constants and pK_a values.

^b From Gibson et al. (1964).

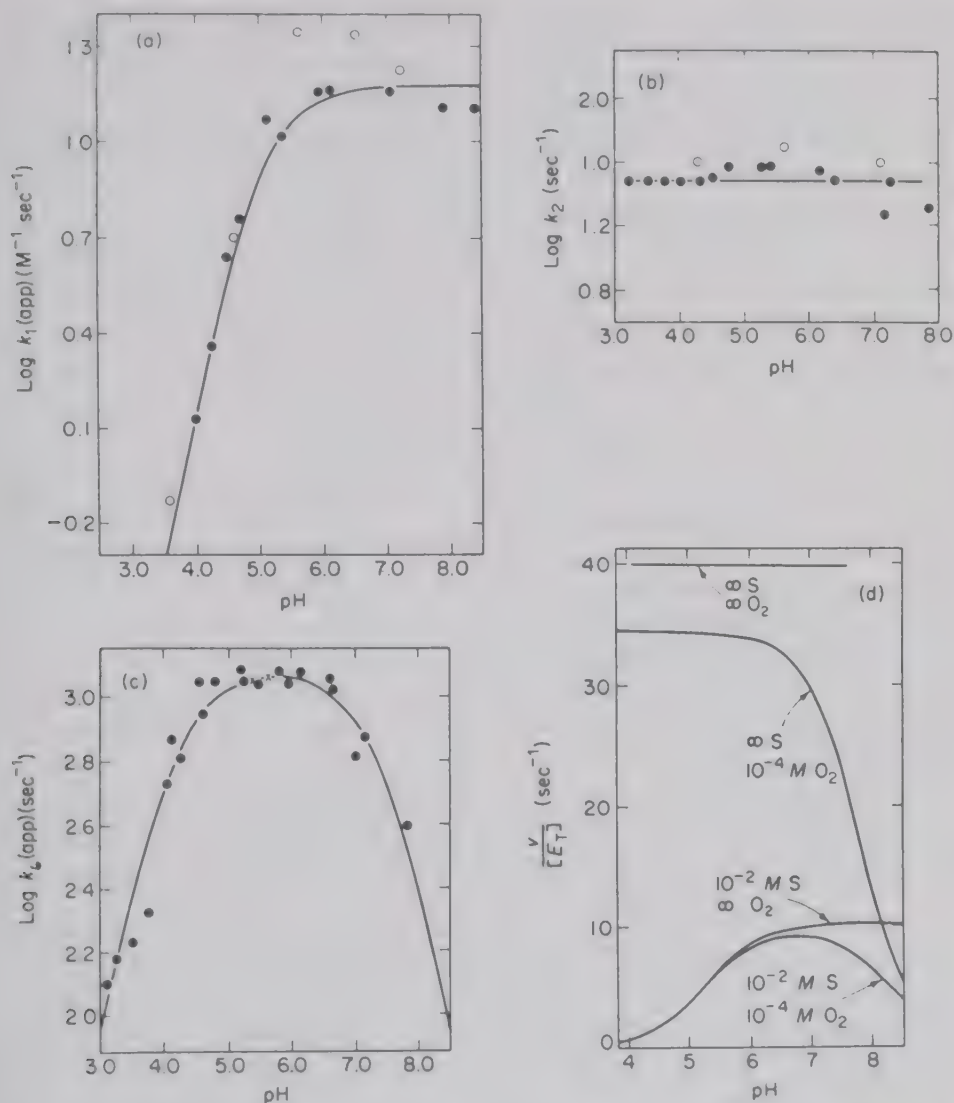


FIG. 8.15. Effect of pH on the rate of oxidation of (a) D-mannose; (b) and (d) 2-deoxy- β -D-glucose; and (c) β -D-glucose by glucose oxidase at 25°C and ionic strength of 0.25. (○), reductive half reaction; (●), spectrophotometric turnover experiments; X, stopped flow turnover experiments. (a), (b), and (c) are plotted by the Dixon method (Dixon and Webb 1958). (d) The pH dependence of the initial turnover rate, v_0 , predicted for various concentrations of 2-deoxy- β -D-glucose and O_2 based on the data of Table 8.5.

From Bright and Appleby (1969); Reprinted from Whitaker (1972), p. 568, courtesy of Marcel Dekker, Inc.

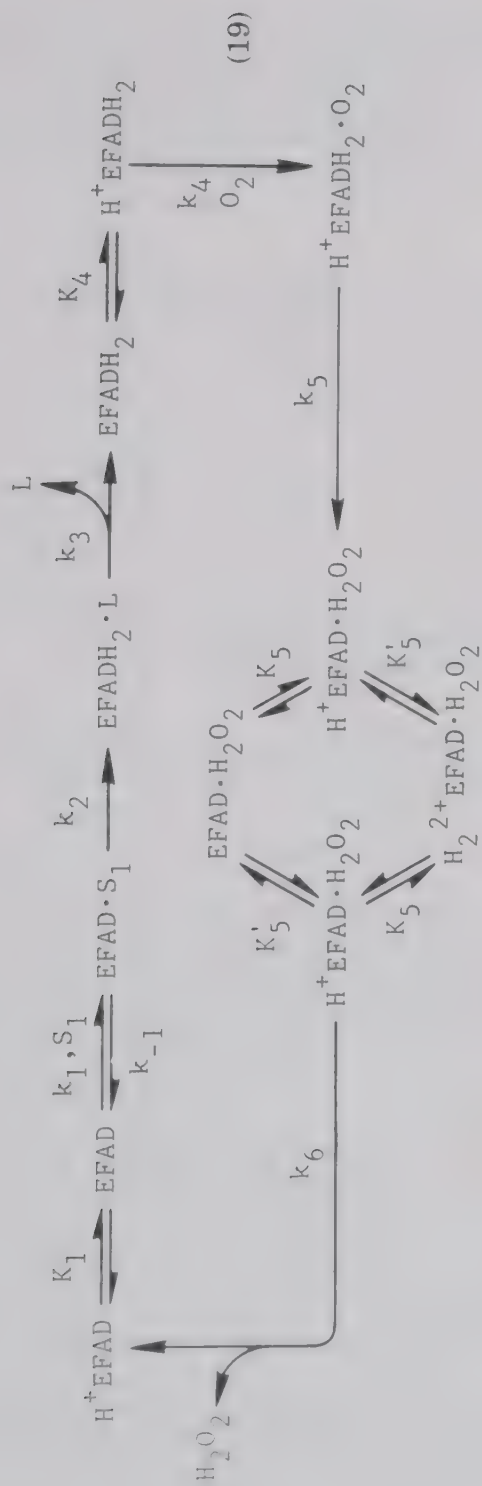
$\text{H}^+ \text{EFAD} \cdot \text{H}_2\text{O}_2$, not from $\text{H}_2^{2+} \text{EFAD} \cdot \text{H}_2\text{O}_2$ or $\text{EFAD} \cdot \text{H}_2\text{O}_2$ (Eq. 19; Table 8.5, $\text{p}K_5$ and $\text{p}K'_5$). Since the release of H_2O_2 from the enzyme $\cdot \text{H}_2\text{O}_2$ complex (k_6) is controlled by two prototropic groups, the pH-V_{max} profile is bell shaped (Fig. 8.15c). Since the rate-controlling step is release of H_2O_2 from $\text{H}^+ \text{EFAD} \cdot \text{H}_2\text{O}_2$, all the prior steps can be elucidated by transient and/or steady-state kinetics.

The rate-determining step for the oxidation of 2-deoxy- β -D-glucose is the oxidation of the substrate to the lactone (Eq. 19). This reaction is $\text{EFAD} \cdot \text{G} \xrightarrow{k_2} \text{EFADH}_2 \cdot \text{L}$ (where G = glucose and L = lactone). As shown in Fig. 8.15b, this step is pH independent (and no $\text{p}K_a$ is given in Table 8.5). The k_2 for 2-deoxy- β -D-glucose is 40 sec^{-1} , compared with 3400 sec^{-1} for β -D-glucose (Table 8.5).

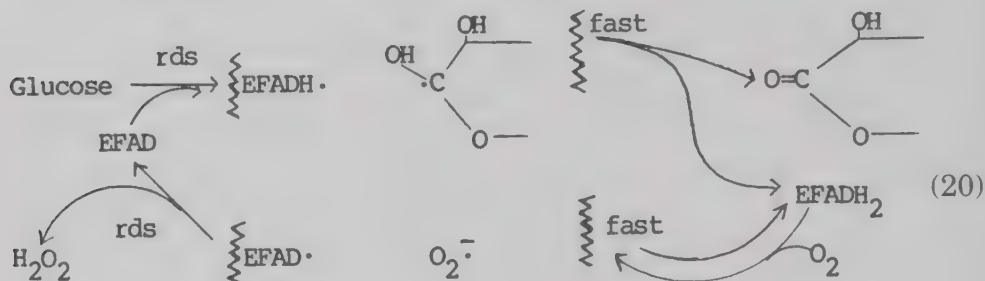
The rate-determining step for the oxidation of D-mannose is its binding to glucose oxidase (Table 8.5). The k_1 for D-mannose is only $15 \text{ M}^{-1} \text{ sec}^{-1}$, compared to $12,600 \text{ M}^{-1} \text{ sec}^{-1}$ for β -D-glucose. This low rate of enzyme-substrate binding is indicative of a slow conformational change needed to accommodate the change in configuration of the C-2 OH group. Binding of substrates to glucose oxidase is controlled by a prototropic group of $\text{p}K_a$ 5.00–5.35 (Table 8.5; Fig. 8.15a). Only the unprotonated enzyme, EFAD, can bind substrate, not $\text{H}^+ \text{EFAD}$ (Eq. 19).

The binding of O_2 to the reduced enzyme is dependent upon the protonation of a group with a $\text{p}K_a$ of 6.90 (Eq. 19; Table 8.5). As shown in Equation 19, the oxidation of β -D-glucose to δ -D-gluconolactone ($\text{EFAD} \cdot \text{G} \xrightarrow{k_2} \text{EFADH}_2 \cdot \text{L}$) is shown as a single step, indicating that the two-electron oxidation occurs in a single step. This is in conformity with failure to observe free radical formation with β -D-glucose, 2-deoxy- β -D-glucose, and D-mannose. More recently, Porter *et al.* (1972) and Chan and Bruice (1977) have reexamined this question with other substrates. Porter *et al.* (1972) found that when the nitroethane anion is mixed with glucose oxidase, under anaerobic conditions, there is a rapid first-order decrease in absorbance at 450 nm (due to disappearance of EFAD) and a concomitant increase in absorbance at 550 nm, indicating formation of the semiquinone form ($\text{EFADH} \cdot$). The ESR spectrum and the pH dependence of the optical spectrum substantiated this. However, the authors' data indicate that when all the enzyme is reduced under anaerobic conditions, only 35% is in the semiquinone form, $\text{EFADH} \cdot$; the other 65% is in the reduced form, EFADH_2 . The authors conclude that the enzyme is partitioned between the two pathways.

Chan and Bruice (1977) found their results to be consistent with a two-step semiquinone intermediate for $\text{EFAD} \cdot \text{S}_1 \xrightarrow{k_2} \text{EFADH}_2 \cdot \text{P}_1$ for-



mation (Eq. 19) when furoin was used as substrate, but not for dihydroxyacetone, glyceraldehyde, or phenacyl alcohol. On the basis of their data for furoin, they proposed that the k_2 and k_5 steps (Eq. 19) could well be written as shown in Eq. 20, reflecting the formation of a free radical semiquinone intermediate at both steps.



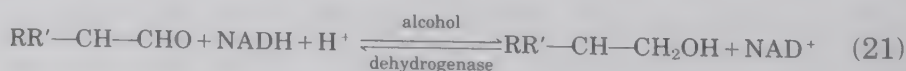
Still needed is direct proof that the oxidation of a good substrate, such as β -D-glucose, proceeds through two one-electron steps via a free radical semiquinone intermediate.

ALCOHOL DEHYDROGENASE AND ALDEHYDE DEHYDROGENASE

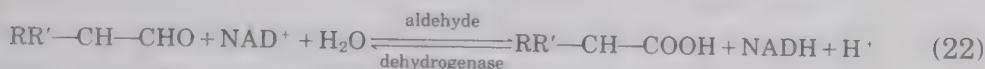
Introduction

Aldehydes can be produced from the action of lipoxygenase on polyunsaturated fatty acids (see section on lipoxygenase, this chapter; Eskin *et al.* 1977) as well as during regular metabolism in plants and animals. In part, these aldehydes are unwanted as they may cause off-flavors in soybeans, for example. Chiba *et al.* (1979) and Takahashi *et al.* (1979) have proposed the use of aldehyde oxidase (aldehyde:oxygen oxidoreductase, EC 1.2.3.1; see section on xanthine oxidase, etc.) to oxidize the aldehydes to acids, with loss of odor. The same oxidation to acids can be accomplished by aldehyde dehydrogenase (aldehyde:NAD⁺ oxidoreductase, EC 1.2.1.3). Conversion of the aldehydes to acids and alcohols and subsequent ester formation is quite important in the flavor development in several fruits (Schwimmer 1982). Formation of ethanol during fermentation of wine is a major industry.

Primary and secondary aldehydes can be reduced by alcohol dehydrogenases to primary and secondary alcohols (Eq. 21).



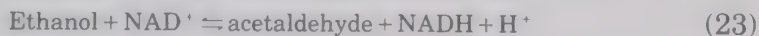
They can also be oxidized by aldehyde dehydrogenases to acids (Eq. 22).



Alcohol Dehydrogenases

Plant Alcohol Dehydrogenases. There is little information on the mechanism of action of alcohol dehydrogenases from higher plants. Stiborová and Leblová (1979) and Čerovská and Leblová (1981) have reported on alcohol dehydrogenases isolated from germinating rape seeds and from germinating pea seeds, respectively.

Both enzymes catalyzed the interconversion of alcohols to aldehydes, as shown in Eq. 23 for ethanol.

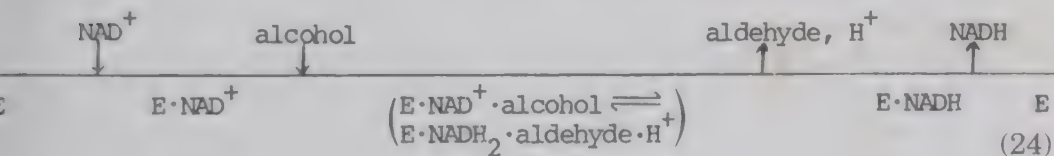


The overall equilibrium constant,

$$K_{\text{eq}} = \frac{[\text{Acetaldehyde}][\text{NADH}][\text{H}^+]}{[\text{Ethanol}][\text{NAD}^+]}$$

was $3.7 \times 10^{-11} M$ at 25°C for the rape alcohol dehydrogenase and $1.7 \times 10^{-11} M$ at 25°C for the pea alcohol dehydrogenase. These values are similar for horse liver alcohol dehydrogenase of $0.98 \times 10^{-11} M$ at 25°C reported by Backlin (1958). Therefore, like most alcohol dehydrogenases, the direction of the equilibrium favors alcohol and NAD^+ .

Kinetically, both enzymes followed an ordered bi-bi mechanism in which the cofactors must bind before either the alcohol or aldehyde. This is shown schematically by the Cleland nomenclature in Eq. 24.



The pH optimum depended on the direction of the reaction. For the rape alcohol dehydrogenase, the pH optimum for reduction of aldehyde to alcohol was near 7 while the pH optimum for oxidation of alcohol to aldehyde was 8.5. Differences in pH optima for direction of reaction have been shown for several other dehydrogenases.

Higher alcohols and aldehydes also were substrates for these enzymes.

Horse Liver Alcohol Dehydrogenase. One of the best studied alcohol dehydrogenases is that from horse liver. It has a molecular weight of 73,000 and consists of two identical subunits with completely independent active sites. It is a Zn(II)-containing enzyme. It catalyzes the reversible oxidation of a variety of primary and secondary alcohols to their respective aldehydes (Sund and Theorell 1963). The equilibrium constant of $9.8 \times 10^{-12} M$ (25°C; Backlin 1958) favors alcohol and NAD^+ formation.

Steady-state kinetics follow an ordered bi-bi mechanism for both directions (Eq. 21).

Structural Features

The complete primary structure and the X-ray crystallographic structure of the enzyme is known to 2.4 Å (Eklund *et al.* 1976). As shown in Fig. 8.16, the enzyme contains two subunits each with two clearly separated domains. One of these domains binds the coenzyme (NAD^+ or $NADH$); the second larger domain binds the zinc atom of the subunit. The two subunits are linked by interaction between the coenzyme binding domains. The catalytic sites are in the junctions between the two domains. The active site has a hydrophobic pocket (barrel in Fig. 8.16) in which the distal end of the alcohol is located (Fig. 8.17).

As shown by Argos *et al.* (1978), the Zn atom in the active site is coordinated to the protein by ligands involving S of Cys-46 and Cys-174 and N of His-67 (Fig. 8.18). A water molecule, or hydroxide ion depending on pH, is the fourth ligand to the Zn(II).

The substrate binding pocket contains three distinct parts (Fig. 8.16). There is a hydrophobic bottom, a hydrophobic barrel through which the substrate must enter, and a rim where both polar and nonpolar residues are present. The bottom part contains Zn(II) coordinated to a water molecule, the nicotinamide part of the coenzyme and Thr-178, and a general acid-base system consisting of the hydroxyl group of Ser-48 and the imidazole group of His-51. The oxygen atom of the Zn(II) bound water molecule forms a hydrogen bridge to the Ser-48 oxygen which in turn is hydrogen bonded to nitrogen of His-51.

Mechanism

The active-site region of alcohol dehydrogenase is shown again in Fig. 8.19, where the addition and orientation of the alcohol is now shown. The mechanism suggested (Brändén *et al.* 1975) is based on electrophilic catalysis mediated by the active site zinc atom. Alcohol

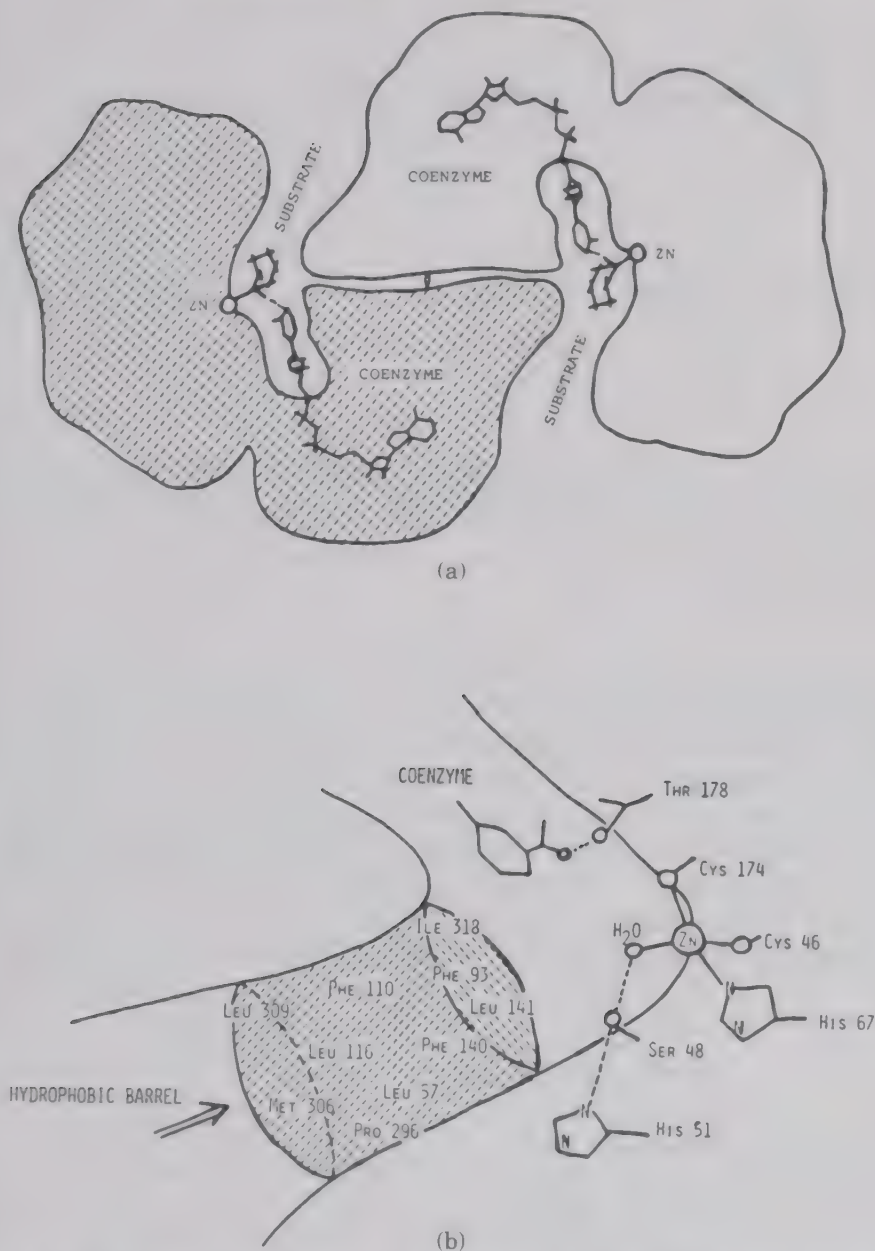


FIG. 8.16. (a) Schematic diagram of a section through the dimeric horse liver alcohol dehydrogenase (LADH) molecule with position of bound coenzyme and cyclohexanol (substrate bound to Zn) shown. (b) Schematic diagram of the active site of LADH as observed by X-ray studies.

Reprinted from Brändén (1977).

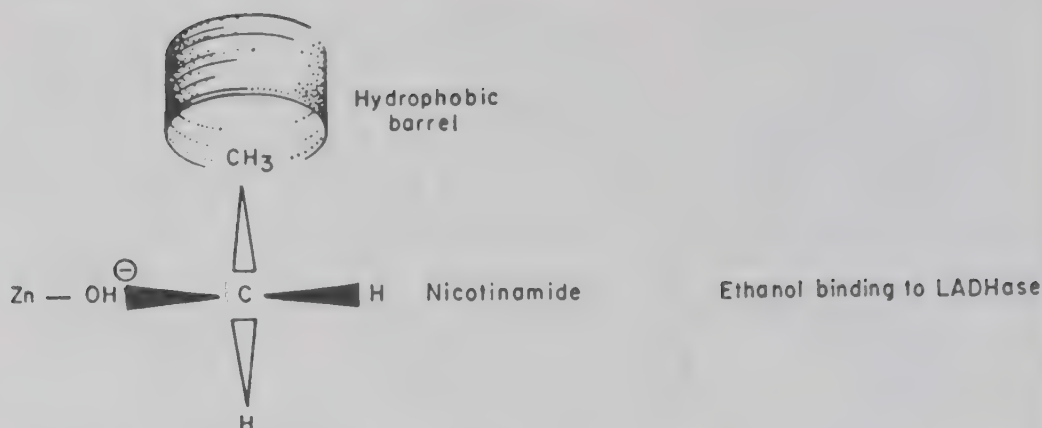


FIG. 8.17. Diagrammatic representation of functionally equivalent groups around the substrate for horse liver alcohol dehydrogenase. The substrate, in the center, is ethanol.

From Argos et al. (1978); With permission from the J. Mol. Biol., Vol. 126, p. 144. © 1978, Academic Press Inc. (London) Limited.

binds directly to zinc as the alcoholate anion (Fig. 8.19a). Formation of the alcoholate ion is facilitated by the hydroxide ion on the zinc, which is preformed from the water on binding of NAD^+ . The hydroxyl ion combines with the hydroxyl hydrogen atom of alcohol to produce water and alcoholate ion. Binding of the alcohol and removal of the hydrogen is probably facilitated by the coordinated system of the hydroxyl group of Ser-48 and nitrogen of His-51. The α -carbon hydrogen is then stereospecifically abstracted as a hydride ion via coordination with the 4 position of the nicotinamide ring of NAD^+ .

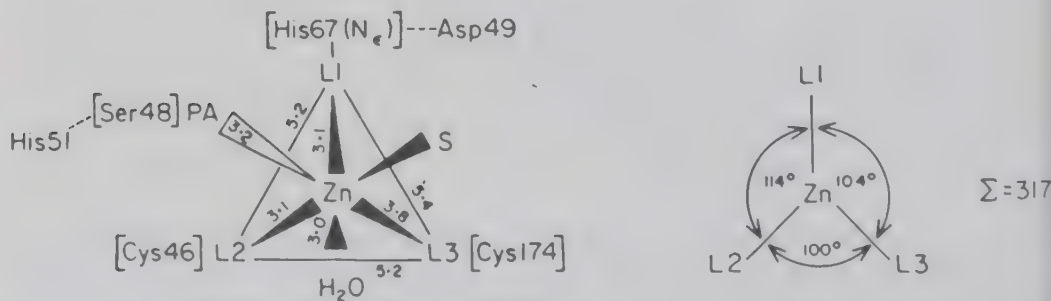


FIG. 8.18. Diagrammatic representation of Zn environment in horse liver alcohol dehydrogenase. The three ligand groups, His-67, Cys-46, and Cys-174, are labeled on right as L_1 , L_2 , and L_3 . PA is the proton abstracting group and S is the substrate binding group to the Zn.

From Argos et al. (1978); With permission from the J. Mol. Biol., Vol. 126, p. 157. © 1978, Academic Press Inc. (London) Limited.

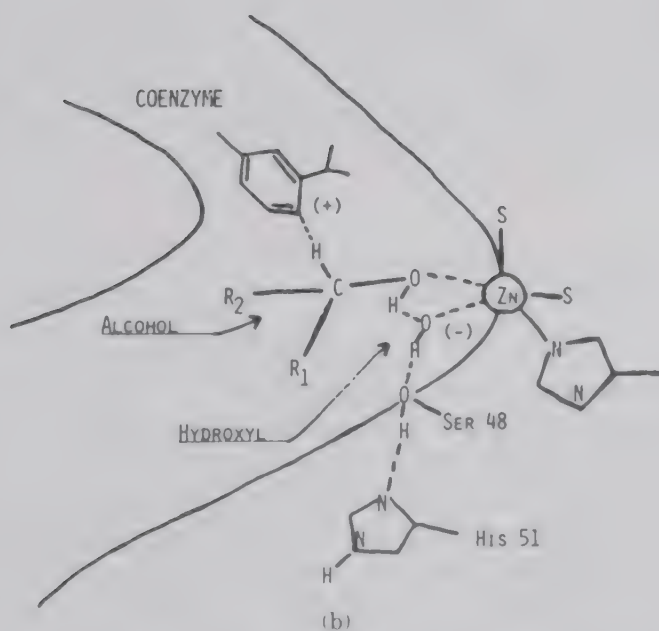
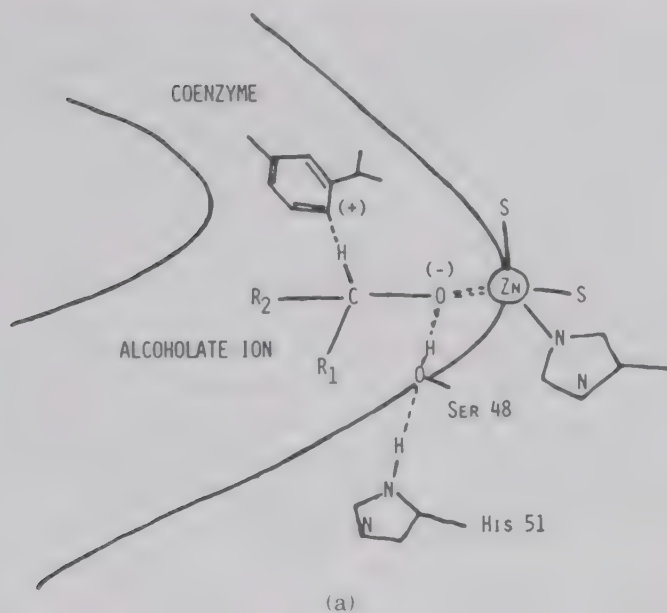


FIG. 8.19. Schematic diagram of productive horse liver alcohol dehydrogenase-substrate complex ($\text{LADH} \cdot \text{NAD}^+ \cdot \text{ethanol}$) as suggested by (a) Brändén *et al.* (1975) and (b) Dworschack and Plapp (1977).

Reprinted from Brändén (1977).

Dworschack and Plapp (1977) have suggested a modification of the mechanistic scheme above (Fig. 8.19b). In this proposal, the hydroxyl hydrogen is not removed as described above, but is removed simultaneously with the removal of the hydride ion from the α -carbon position by the nicotinamide part of the cofactor in a concerted general acid-general base catalysis. The zinc-bound hydroxide ion is postulated to be the general base catalyst for the oxidation of alcohols and the zinc-bound water molecule as the general acid catalyst for the reduction of aldehydes.

Greeves and Fink (1980), using low-temperature techniques (-45°C), have shown that the binding of NADH to horse liver alcohol dehydrogenase occurs in three steps, the second-order combination of enzyme and NADH followed by two first-order steps, apparently reflecting conformational changes as the enzyme and NADH better accommodate to each other in the binary complex.

Aldehyde Dehydrogenase

The reaction catalyzed by this enzyme is shown in Eq. 22.

Aldehyde dehydrogenase has been much less studied than has alcohol dehydrogenase. Purification and properties of aldehyde dehydrogenases from horse liver (Feldman and Weiner 1972; Eckfeldt and Yonetani 1976), human liver (Sidhu and Blair 1975A, B), and sheep liver (Crow *et al.* 1974; MacGibbon *et al.* 1977A, B; Hart and Dickinson 1978; Buckley and Dunn 1982; Buckley *et al.* 1982) have been reported.

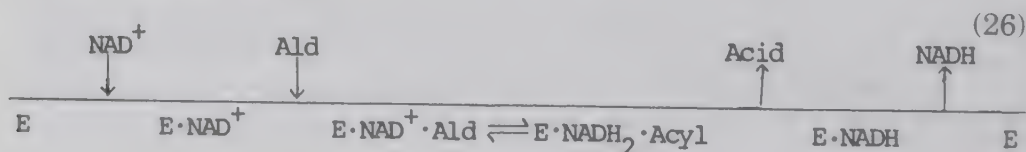
Kinetics

The overall reaction catalyzed is that shown in Eq. 25.



The overall reaction, with a pH optimum of 7.6, is irreversible. The maximum values for both acetaldehyde and propionaldehyde are 0.25 sec^{-1} at pH 7.6 and room temperature. The reaction is very slow compared to $30\text{--}100 \text{ sec}^{-1}$ for most hydrolytic enzymes and up to $10^6\text{--}10^7 \text{ sec}^{-1}$ for oxidoreductases such as peroxidase and catalase (see above).

The reaction follows an ordered bi-bi mechanism which can be diagrammed by the Cleland method (Eq. 26; MacGibbon *et al.* 1977A; Buckley *et al.* 1982).



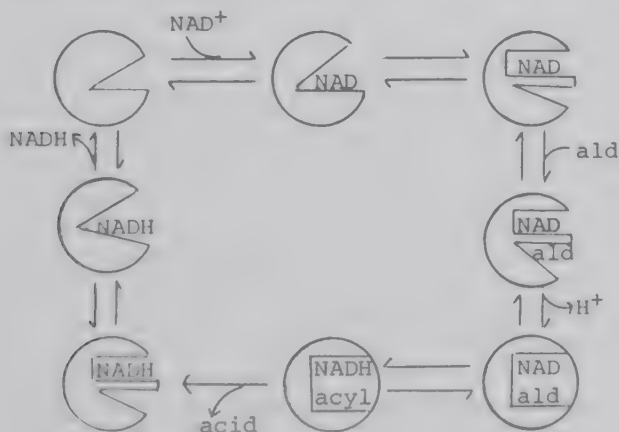
The reaction is complicated by a lag phase, with length dependent on enzyme concentration, nonlinear double-reciprocal plots with aldehyde as substrate, substrate activation, and irreversibility of the reaction, rather an oddity for NAD^+/NADH -requiring enzymes.

Mechanism

Based on detailed studies, including proton burst determinations (Buckley *et al.* 1982) and rapid-scanning UV-visible spectroscopy (Buckley and Dunn 1982), the mechanism appears to be that described in Scheme 6. The resting enzyme binds, in ordered sequence, NAD^+ to give the binary $\text{E} \cdot \text{NAD}^+$ complex whereupon it undergoes a conformational change forming the aldehyde binding site. The aldehyde binds to give the ternary complex, $\text{E} \cdot \text{NAD}^+ \cdot \text{ald}$, which undergoes a second conformational change and the release of a proton. The new conformer, $\text{E} \cdot \text{NAD}^+ \cdot \text{ald}$, undergoes a chemical reaction to form $\text{E} \cdot \text{NADH} \cdot \text{acyl}$. In an irreversible step, the acid is released to leave the binary complex $\text{E} \cdot \text{NADH}$, which undergoes a third conformational change (reversal of the second conformational change). The $\text{E} \cdot \text{NADH}$ binary complex undergoes a fourth conformational change (reversal of the first conformational change) to close the ald, acyl binding site and later the NADH binding site, and NADH is then released to return to the resting enzyme form.

Buckley *et al.* (1982) have proposed that the rate-determining step in the reaction is the conformational change following formation of the ternary $\text{E} \cdot \text{NAD} \cdot \text{ald}$ complex, resulting in release of a proton (Scheme 6).

Tsai and Sher (1980) have shown that crystalline horse liver alco-



SCHEME 6

hol dehydrogenase also has aldehyde dehydrogenase activity, leading to acid formation. Several types of data were presented to show that both activities are associated with the same protein molecule. k_{cat} for octanol dehydrogenation to octanal at pH 9.0 (optimum) and 37°C was reported to be $1.87 \times 10^3 \text{ min}^{-1}$ compared to 13.4 min^{-1} for octanal dehydrogenation to octanoic acid at pH 7.4 (optimum) and 37°C. The ratio of k_{cat} (alcohol dehydrogenase)/ k_{cat} (aldehyde dehydrogenase) was 140. The difference in pH optimum of activity for alcohol dehydrogenase and aldehyde dehydrogenase activities permitted the activities to be studied separately. Interestingly, Arrhenius activation energies, E_a , are different for the two activities, implying either a change in kinetic mechanism or in rate-determining step. At high octanal concentrations, the Arrhenius plot was nonlinear for aldehyde dehydrogenase activity, giving E_a of 18.3 kcal/mol between 15°–20°C and 10.4 kcal/mol between 32°–40°C. The Arrhenius plot for alcohol dehydrogenase activity (starting with octanol and NAD^+) was linear, with E_a of 4.52 kcal/mol.

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Oxidation of Lipids in Biological Tissue and Its Significance

*H.W. Gardner*¹

THE POLYUNSATURATED FATTY ACID CASCADE

Both plants and animals possess biochemical systems to sequentially transform glyceride lipid into fatty acid derivatives by a process that could be given the generic name, "polyunsaturated fatty acid (PUFA) cascade." Although similarities exist between the plant and animal cascade, substantial differences exist not only in the pathways of the sequence, but also in the control mechanisms regulating the pathways and the so-called end products formed. Typically, the cascade is composed of the following steps: (a) hydrolysis of glyceride lipids by phospholipase, lipase, or other lipolytic acyl hydrolases; (b) oxidation of the released PUFA by lipoxygenase (LOX) or prostaglandin endoperoxide synthetase; and (c) enzymatic conversion of these oxidized fatty acids (either fatty acid hydroperoxides or fatty acid hydroperoxyendoperoxides) into a variety of fatty acid derivatives. The compounds produced by the cascade usually play an indispensable role in controlling certain metabolic functions of organisms.

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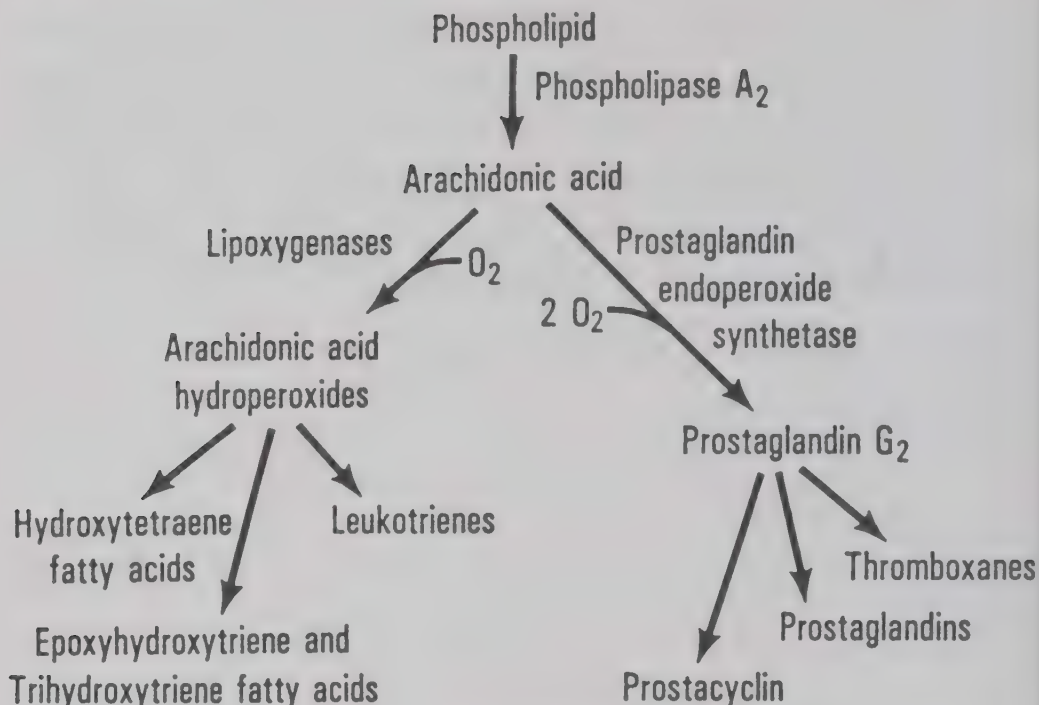


FIG. 9.1. Mammalian arachidonic acid cascade.

Arachidonic Acid Cascade

The mammalian arachidonic acid cascade has been the subject of intense research for the past two decades, and Fig. 9.1 summarizes the known pathways. The arachidonic acid cascade as well as the physiological effects of the compounds formed have been described in numerous specialized reviews; however, recent reviews by Nelson *et al.* (1982) and Marcus (1978) give succinct overviews of most aspects of the field.

The Linoleic/Linolenic Acid Cascade

The principal PUFAs indigenous to plant lipid are linoleic and linolenic acids, and consequently, the cascade enzymes utilize these fatty acids as substrates. Figure 9.2 summarizes the known pathways of the plant linoleic/linolenic acid cascade. For details, a number of reviews can be consulted (Galliard 1975; Veldink *et al.* 1977; Eskin *et al.* 1977; Gardner 1980). Unlike the mammalian arachidonic acid cascade, less is known of the physiological significance of the linoleic/linolenic acid cascade; however, recent research has provided some

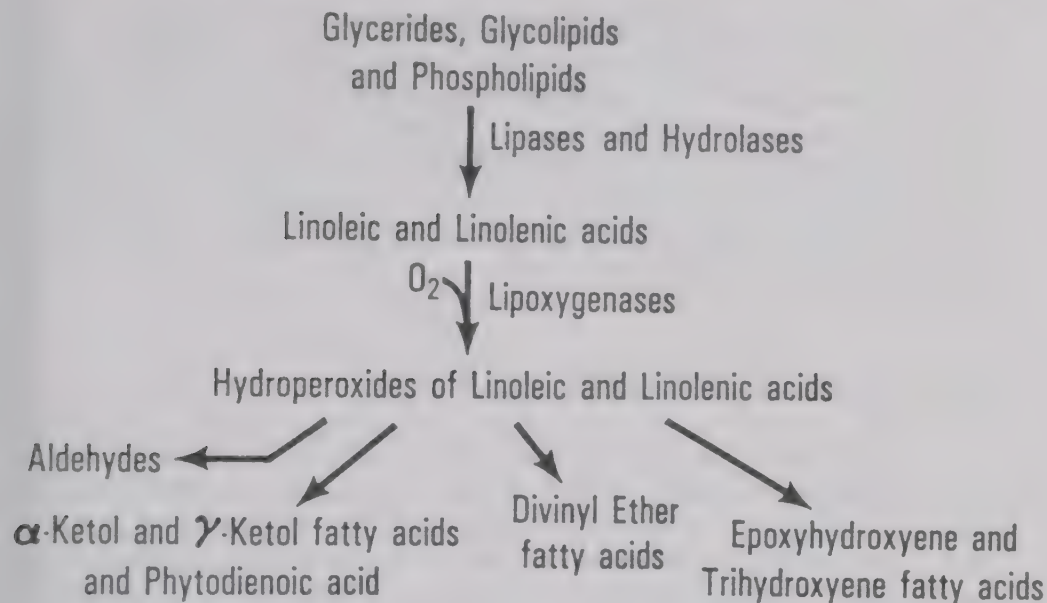


FIG. 9.2. Linoleic/linolenic acid cascade of plants.

interesting results. The potential significance of the cascade to plants will be discussed in the final section of this chapter.

Significance of the Cascade to Food Quality

Although the physiological aspects of the arachidonic acid cascade in animals have received the most attention, its effect on meat quality is not specifically known. The well-known “warmed-over” flavor of meats occurs after storage of cooked meats; thus, the cause may be due to oxidation of lipids by denatured heme proteins rather than the enzymes of the cascade. In contrast, the linoleic/linolenic acid cascade in plants has been directly linked to the development of off-flavors in vegetables and fruit. Since the cascade-derived aldehydes and the corresponding alcohols originating from the aldehydes impart characteristic fresh flavors to fruit and vegetables, the term “off-flavor” may be improper. Aldehydes originate from hydroperoxide lyase action on linoleic and linolenic acid hydroperoxides, and alcohols arise from the aldehydes by reduction via alcohol dehydrogenase. Grassy/beany flavors are attributed to hexanal, hexenal, and their alcohols, whereas nonenal, nonadienal, and their alcohols have odors variously described as melon, cucumber, or violet. These flavor substances become undesirable when they accumulate or cause imbalance in the relative

levels with other flavors. Hydroperoxide lyase and its influence on flavors has been reviewed recently by Gardner (1980; in press).

Bitter tastes have been attributed to cascade products, hydroxy-octadecadienoic acids (Biermann *et al.* 1980), and trihydroxyoctadecenoic acids (Baur and Grosch 1977). These fatty acids have been found in diverse foods and beverages, such as oat flour (Biermann *et al.* 1980), soy products (Moll *et al.* 1979; Baur *et al.* 1977), and beer (Esterbauer and Schauenstein 1977).

FORMATION OF HYDROPEROXIDES

PUFAs are oxidized enzymatically into fatty acid hydroperoxides by both plant and animal LOXs. The enzyme mimics a radical-initiated autoxidation of PUFA. Although both LOX catalysis and autoxidation are believed to be radical processes, they differ both in the mechanism of reaction and products formed. Autoxidation is self-sustained by radical propagation (Fig. 9.3) affording a racemic mixture of hydroperoxides, whereas the iron-active site of LOX catalyzes a one-electron redox cycle with PUFA and O₂ (Vleigenthart 1978; Fig. 9.4), giving rise to hydroperoxides with a high degree of chiral and positional purity. With soybean LOX, the conditions of linoleic acid oxidation can be manipulated to obtain varying ratios of two chiral products, [13S]-13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid and [9S]-9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid (Veldink *et al.* 1970; Christopher *et al.* 1972). When the molecular models of these two chiral products are positioned head to tail with one another, it can be seen that the hydroperoxydiene moieties are spatially identical (Fig. 9.5). Further, the removal of the C-11 hydrogen by LOX is stereospecific (Egmond *et al.* 1972), and the removal is also spatially identical with the arrangement shown in Fig. 9.5. This implies that the positioning of the linoleic acid substrate may occur either head-first or tailfirst at the active site.

PEROXY RADICAL MODEL FOR PROSTAGLANDIN FORMATION

In addition to the oxidation of PUFA by LOX, prostaglandin endoperoxide synthetase from mammals oxidizes PUFA, usually arachidonic acid, into the hydroperoxyendoperoxide, prostaglandin G₂. When the structures of prostaglandins became known, Hamberg and Samuelsson (1967) devised a radical mechanism for their biosynthesis.

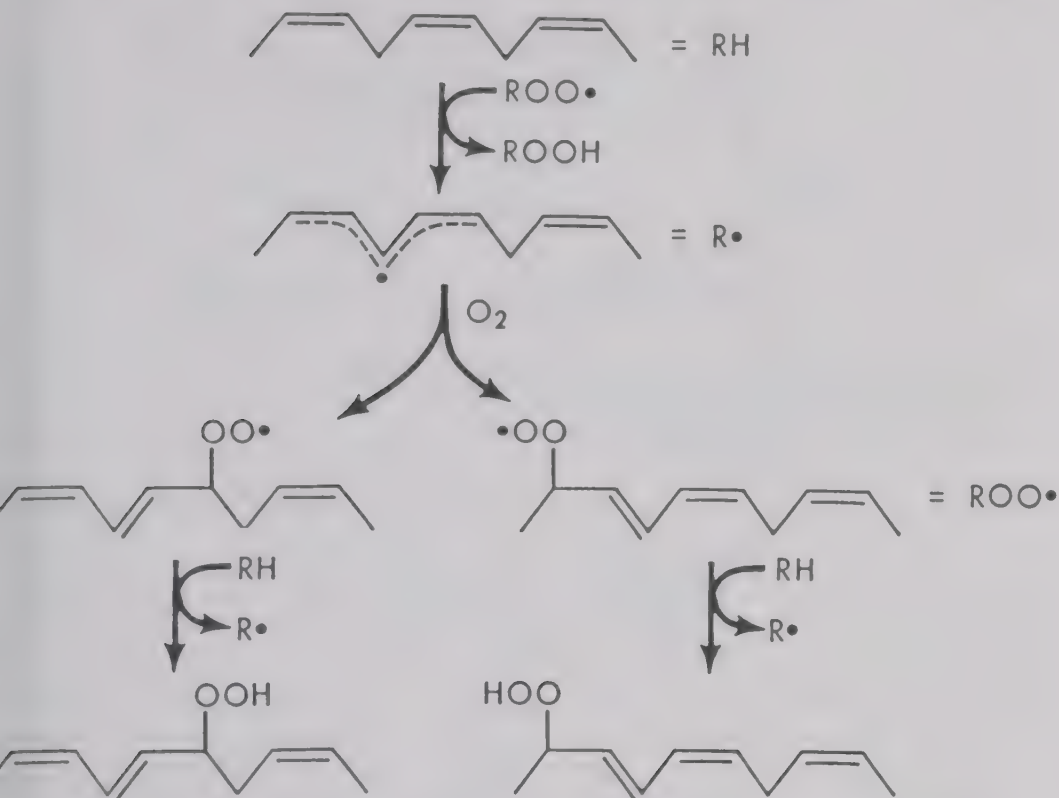


FIG. 9.3. Autoxidation of a polyunsaturated fatty acid. Structures are abbreviated to show only the unsaturation.

Subsequently, a number of workers discovered that prostaglandin-like compounds formed simply by autoxidation of PUFA with three or more double bonds. Formation of these compounds is preceded by autoxidation of PUFA into monohydroperoxides, and subsequent homolytic scission of the monohydroperoxide into a peroxy radical leads to cyclization into endoperoxide. Alternatively, peroxy radicals, which are precursors of monohydroperoxides (Fig. 9.3), can cyclize directly into endoperoxides. Starting with a pure chiral hydroperoxide of linolenic acid, [13S]-13-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid (methyl ester), O'Connor *et al.* (1981) were able to track the stereochemical pathway of the autocatalytic production of prostaglandin-like fatty acids as well as the hydroperoxy cyclic peroxides that also formed (Fig. 9.6). The major difference between the prostaglandin analog and authentic prostaglandin G (aside from the chiral placement of the initial oxidation) was the *cis* configuration of the alkyl chains vs *trans* in the authentic compound.

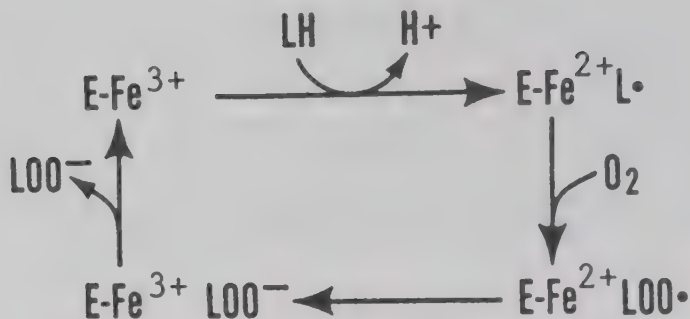


FIG. 9.4. Mechanism of oxidation of linoleic acid (LH) to hydroperoxide anion (LOO^-) by soybean lipoxygenase ($E-Fe^{3+}$).
 From Vliegthart (1978), reprinted from Gardner (1980).

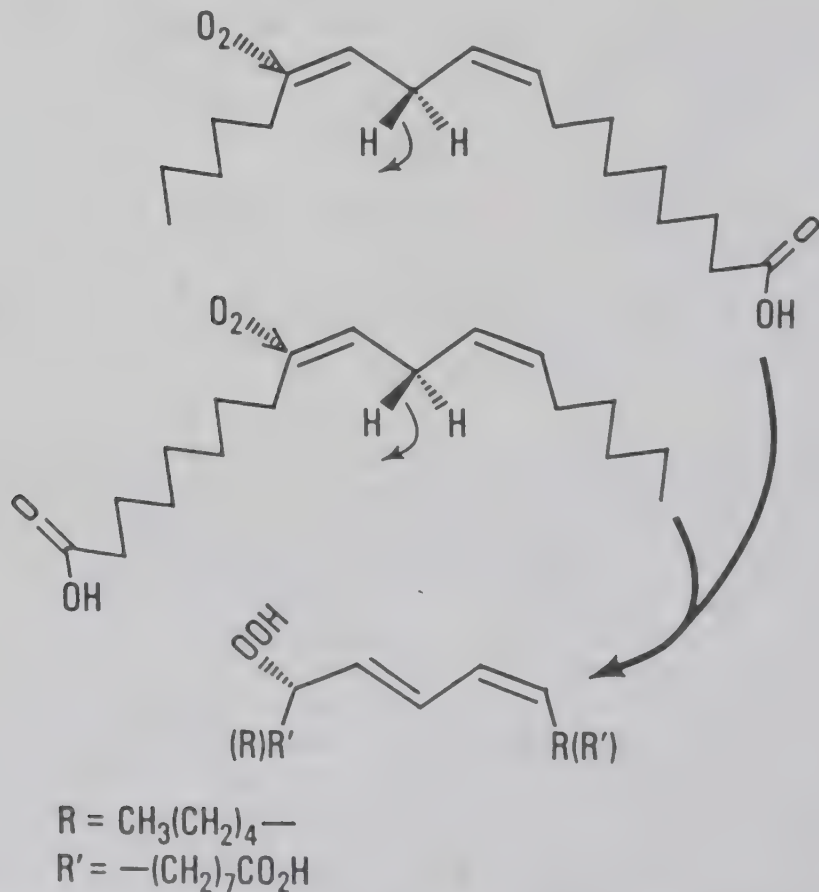


FIG. 9.5. Oxidation of linoleic acid by soybean lipoxygenase showing that the oxidation at C-9 and C-13 as well as the hydrogen removal from C-11 are spatially identical when the molecules are arranged head to tail.

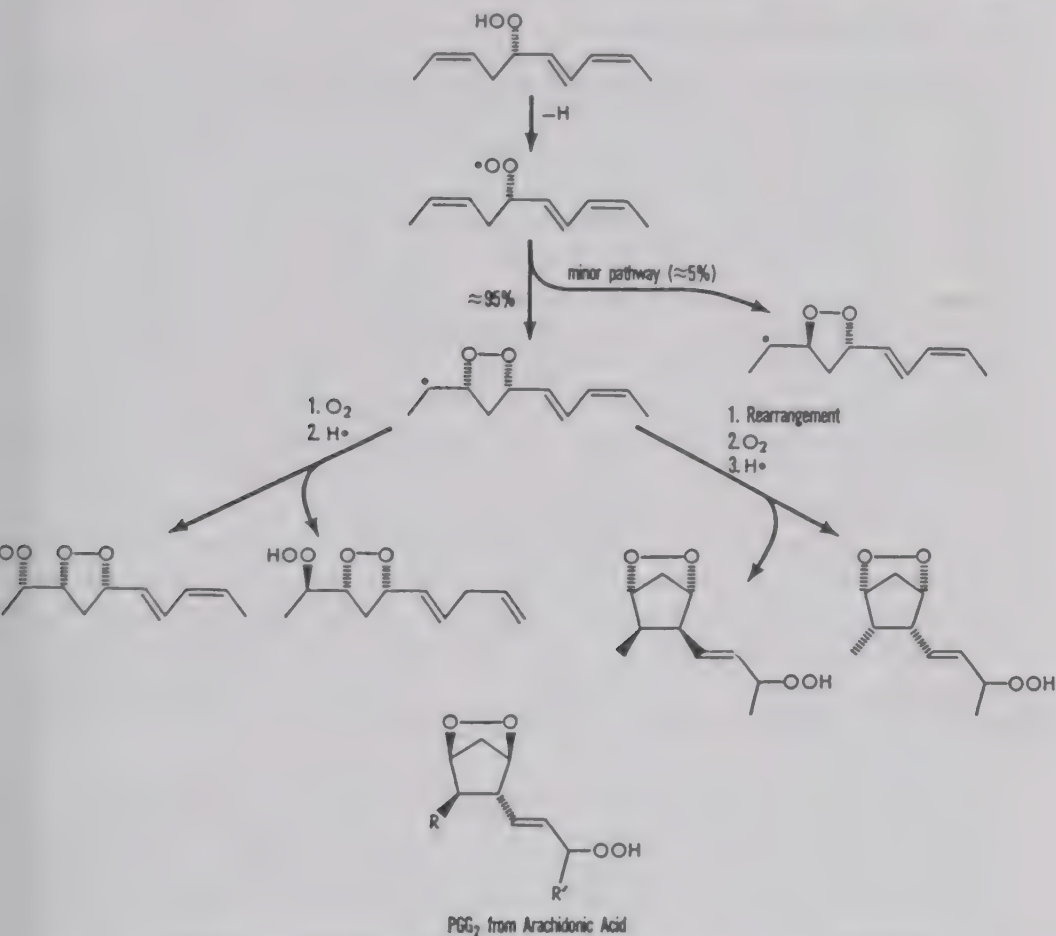


FIG. 9.6. Stereochemistry of formation of hydroperoxy cyclic peroxides and prostaglandin-like endoperoxides from the [13S]-hydroperoxide of linolenic acid (O'Connor *et al.* 1981) and a stereochemical comparison with authentic prostaglandin G_2 from enzymatic oxidation of arachidonic acid. Structures are abbreviated.

Reprinted from Gardner (1983).

POTENTIAL CHEMICAL MODELS FOR BIOCHEMICAL CONVERSION OF HYDROPEROXIDES

With the possible exception of the leukotriene pathway (Fig. 9.1), nearly all the enzymatic reactions of hydroperoxides possibly can be defined by model chemical reactions, such as by the autocatalytic formation of prostaglandin-like substances discussed above.

Oxy Radical Mechanisms

Epoxyhydroxy and Trihydroxy Fatty Acids. Epoxyhydroxy fatty acids containing one or more double bonds are common metabolites of both animal and plant PUFA cascades (Nelson *et al.* 1982; Gardner 1980). Trihydroxy fatty acids are thought to originate from hydrolysis of the epoxyhydroxy fatty acids in enzyme preparations from both plants (Graveland 1970) and animals (Falardeau *et al.* 1976). The epoxyhydroxy fatty acids may arise from alkoxy radicals formed by homolytic scission of the hydroperoxide group. As shown in Fig. 9.7, oxy radicals have a propensity to cyclize with a vicinal double bond. The epoxyallylic radical formed by cyclization can either scavenge O_2 (Gardner *et al.* 1978; Fig. 9.7) or react with a hydroxyl radical to give epoxyhydroxy fatty acids directly (Dix and Marnett 1983). The epoxyhydroperoxy fatty acids from O_2 scavenging commonly degrade further into epoxyhydroxy and epoxyoxo fatty acids (Gardner and Kleiman 1981). Among these compounds the allylic epoxides, such as 12,13-epoxy-9-hydroxy-*trans*-10-octadecenoic acid, are readily hydrolyzed by very mild acid conditions into trihydroxy fatty acid (Gardner, unpublished observations). As shown by Fig. 9.8, there is evidence that epoxide hydrolysis proceeds by both S_N1 and S_N2 mechanisms (Gardner *et al.* 1984A, B).

Although there have been claims for the enzymatic formation of epoxyhydroxy and trihydroxy fatty acids (e.g., Pace-Asciak *et al.* 1983), these compounds also may originate through pseudoenzymatic catalysis. For example, under certain conditions, soybean LOX catalyzed the transformation of 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid into *trans*-12,13-epoxy-11-hydroxy-*cis*-9-octadecenoic acid (Garsen *et al.* 1976), and hemoglobin catalyzed a similar reaction of the

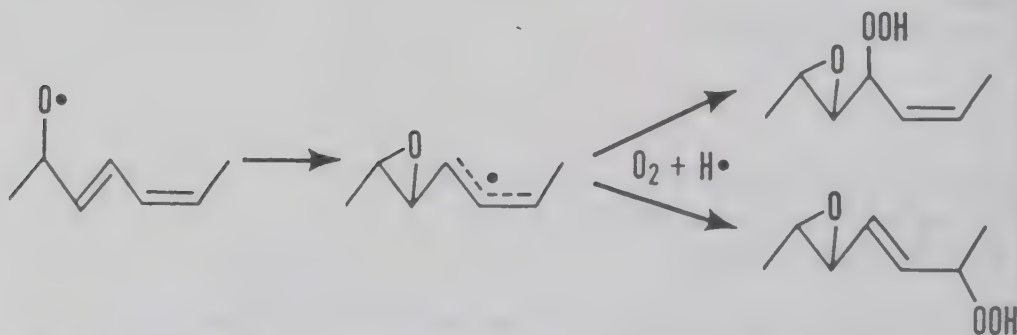


FIG. 9.7. Formation of epoxides by rearrangement of an oxy radical from homolytic cleavage of a linoleic acid hydroperoxide. Structures are abbreviated to show only C-8 through C-14.

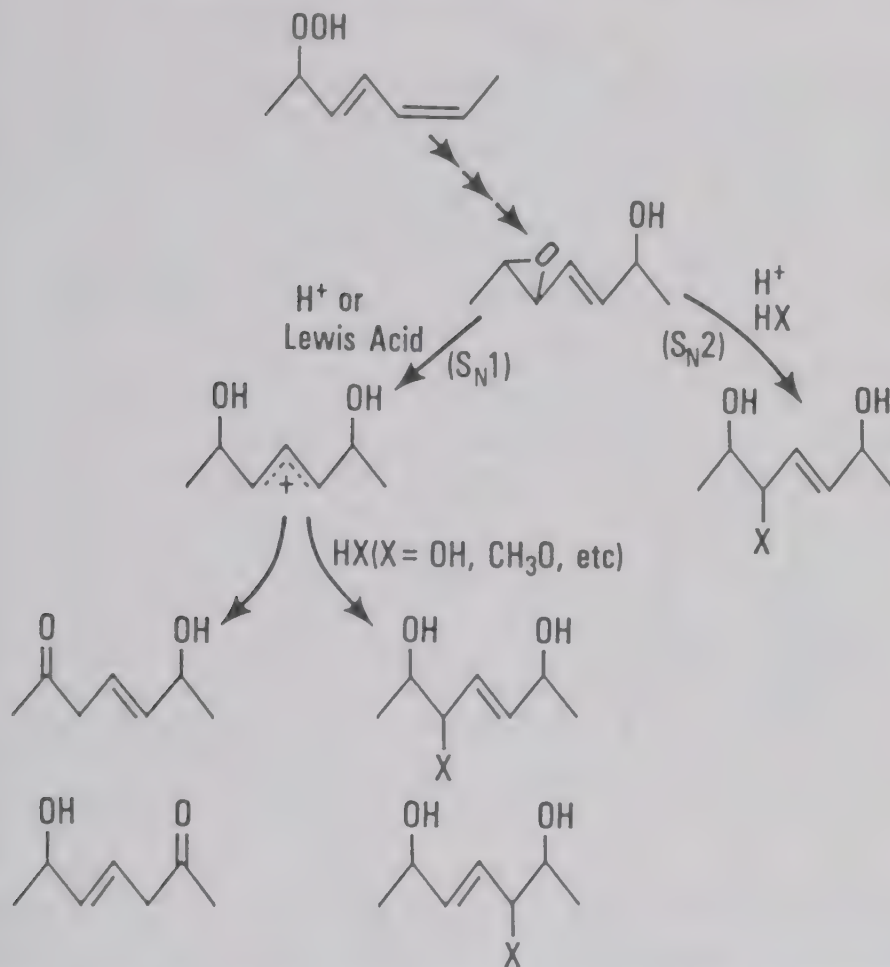


FIG. 9.8. Formation of trihydroxyene and hydroxyoxoene fatty acids from 13-hydroperoxylinoleic acid through an epoxyhydroxyene intermediate. Structures are abbreviated to show only C-8 through C-14. HX is a protic solvent, usually H₂O in biological systems.

hydroperoxide into three isomeric epoxyhydroxyene fatty acids (Hamburg 1975). Both LOX and hemoglobin may have caused an iron-assisted homolysis of the hydroperoxide.

Scission Products. Under anaerobic conditions, incubation of 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid and linoleic acid with soybean LOX caused carbon-chain cleavage of the fatty acid hydroperoxide into both pentane and 13-oxo-9,11-tridecadienoic acid (Garsen *et al.* 1971). Subsequently, a number of workers identified pentane as a product of various LOXs under both aerobic and anaerobic

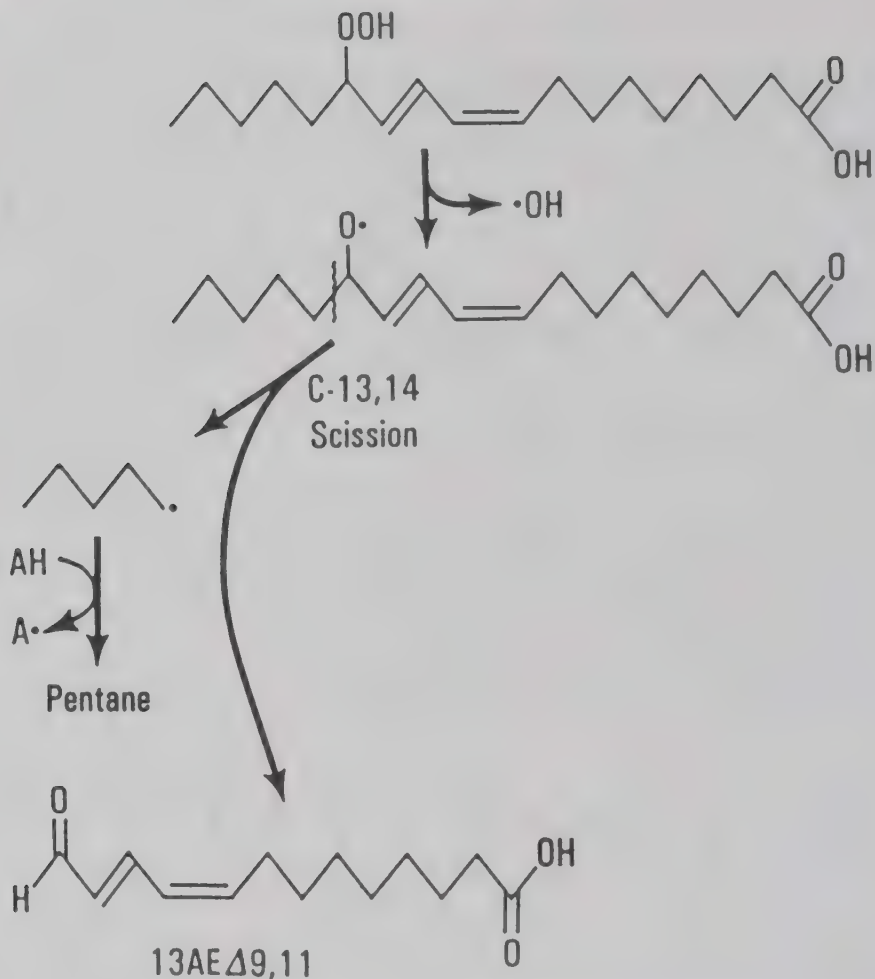


FIG. 9.9. Formation of pentane and 13-oxotridecadienoic acid from 13-hydroperoxylinoleic acid via β -scission of an oxy radical.

conditions. The LOX cleavage of hydroperoxyoctadecadienoic acid resembled the scission obtained by treating hydroperoxides with heat ($\sim 150^{\circ}\text{--}200^{\circ}\text{C}$). It is generally agreed that heat causes homolysis of the hydroperoxide group into an alkoxy radical, and this radical subsequently undergoes β -scission. For the 13-hydroperoxide of linoleic acid, principal products from heat treatment are pentane and 13-oxo-9,11-tridecadienoic acid (Gardner and Plattner 1984; Fig. 9.9). Evidently, the scission is another example of LOX catalyzing a reaction characteristic of those arising from oxy radicals.

Heterolytic Pathways

Hydroperoxide Lyase. As reviewed by Gardner (in press), hydroperoxide lyase from a variety of plants cleaved the hydroperoxides of either linoleic or linolenic acid into aldehydes. The 13-hydroperoxide of both linoleic and linolenic acid is converted into C-6 aldehyde (hexanal or *cis*-3-hexenal from 13-hydroperoxides of linoleic or linolenic acid, respectively) and 12-oxo-*cis*-9-dodecenoic acid. From the 9-hydroperoxides of either linoleic or linolenic acid, 9-oxononanoic acid and a C-9 aldehyde (*cis*-3-nonenal or *cis*-3,*cis*-6-nonadienal from 9-hydroperoxide of either linoleic or linolenic acid, respectively) are produced. Of these lyase products, the β , γ -unsaturated aldehydes are easily isomerized into α , β -unsaturated aldehydes either enzymatically or autocatalytically. The action of lyase affords one of the best examples of an enzymatic reaction that can be reproduced by a chemical model. The heterolytic cleavage of hydroperoxides is readily achieved by a Lewis acid in aprotic solvent (Gardner and Plattner 1984). As illustrated by Fig. 9.10, the aldehydes formed by BF_3 treatment of the 9- or 13-hydroperoxide of methyl linoleate are similar to those expected from lyase activity. Because the acidic conditions promote conversion of β , γ - into α , β -unsaturated aldehydes, the unsaturation was of the latter configuration after BF_3 treatment. A carbon to oxygen rearrangement was proposed for the acid-catalyzed cleavage (Fig. 9.11).

Divinyl Ethers. According to Galliard *et al.* (1973), a potato enzyme transformed the 9-hydroperoxide of linoleic acid into 9-(*trans*-1',*cis*-3'-nonadienyloxy)-*trans*-8-nonenic acid. The 9-hydroperoxide of linolenic acid was also a substrate for the enzyme (Galliard *et al.* 1973), but not the 13-hydroperoxide of either linoleic or linolenic acid (Galliard and Matthews 1975). Since the discovery of the pathway to divinyl ethers from hydroperoxides, their mechanism of formation has remained a mystery. The mechanism of Fig. 9.11 could serve as a plausible route; that is, the hemiacetal, which is the proposed intermediate in the formation of aldehydes, may dehydrate into a divinyl ether. The major problem with this mechanism lies in the evidence that $^{18}\text{O}_2$ -labeled hydroperoxide is not converted into ^{18}O -labeled divinyl ether (Galliard and Matthews 1975). Although this would appear to rule out the pathway outlined in Fig. 9.11, these workers used a 9-hydroperoxide substrate with relatively low levels (10%) of $^{18}\text{O}_2$ label. Additional experiments with H_2^{18}O were not conclusive. It would appear that further research in this area may be appropriate.

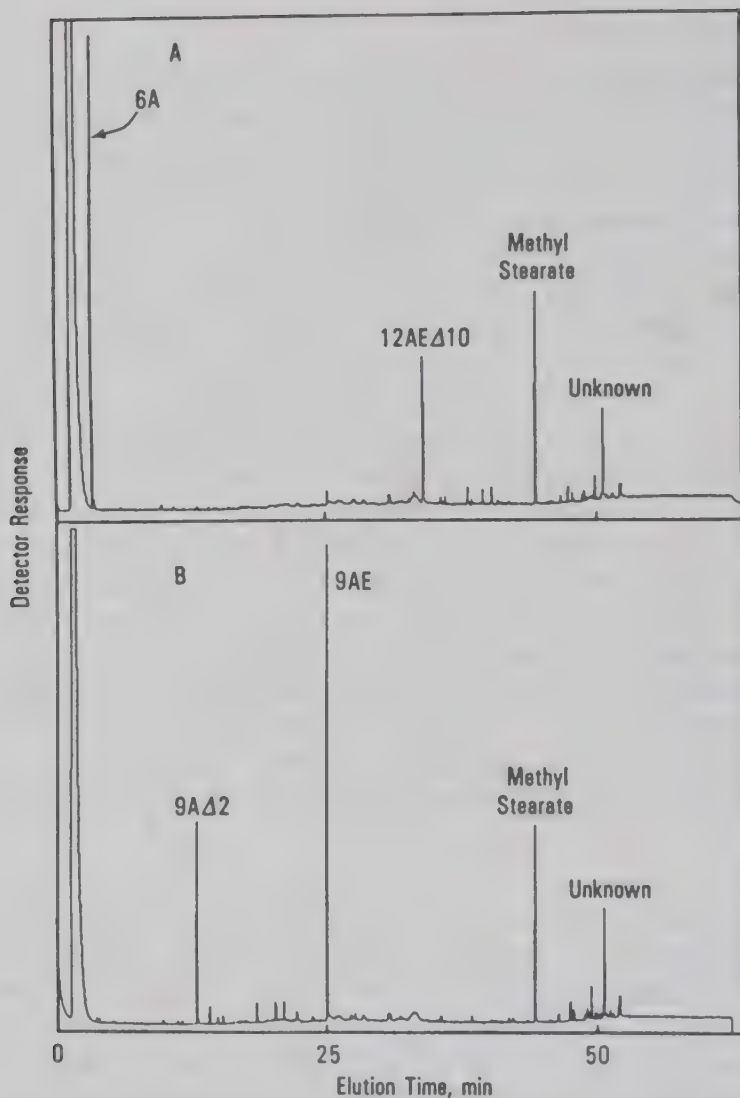


FIG. 9.10. Capillary gas chromatographic separation of aldehydes from heterolytic cleavage of the methyl esters of 13-hydroperoxylinoleic acid (A) and 9-hydroperoxylinoleic acid (B) catalyzed by BF_3 in anhydrous ether. Abbreviations: 6A, hexanal; 12AE Δ 10, methyl 12-oxo-*trans*-10-dodecenoate; 9A Δ 2, *trans*-2-nonenal; and 9AE, methyl 9-oxononanoate. Methyl stearate served as an internal standard.

Reprinted from Gardner and Plattner (1984).

Epoxyhydroxy and Trihydroxy Fatty Acids. The biosynthesis of epoxyhydroxy and trihydroxy fatty acids was discussed in a previous section, and rearrangement of an oxy radical was proposed as a possible initiating event (Fig. 9.7). However, a recent investigation has

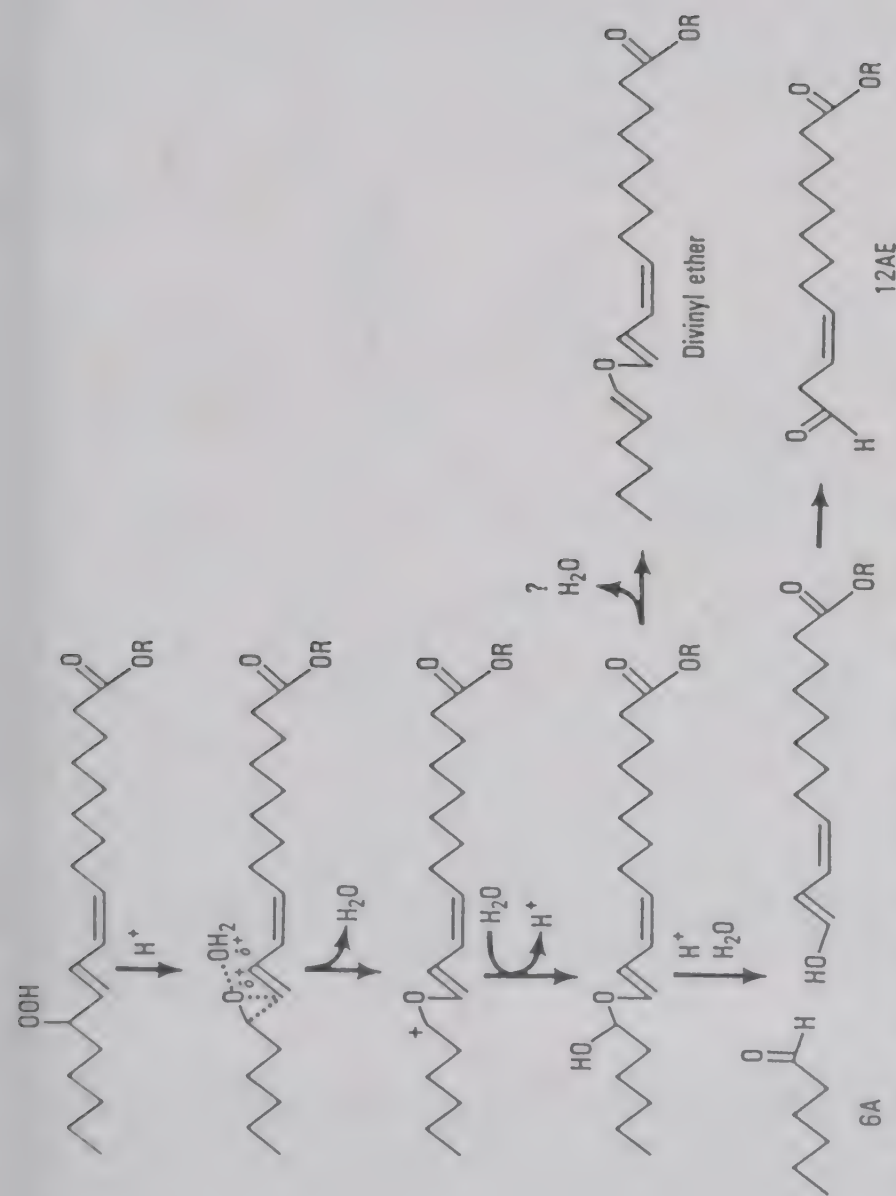


FIG. 9.11. Proposed mechanism of heterolytic cleavage of 13-hydroperoxylinoleic acid (or ester) and a postulated route to divinyl ethers. Although a proton is shown as the catalyst, the Lewis acid (BF_3) in aprotic solvent was much more efficient in producing aldehydes.

Reprinted in part from Gardner and Plattner (1984).

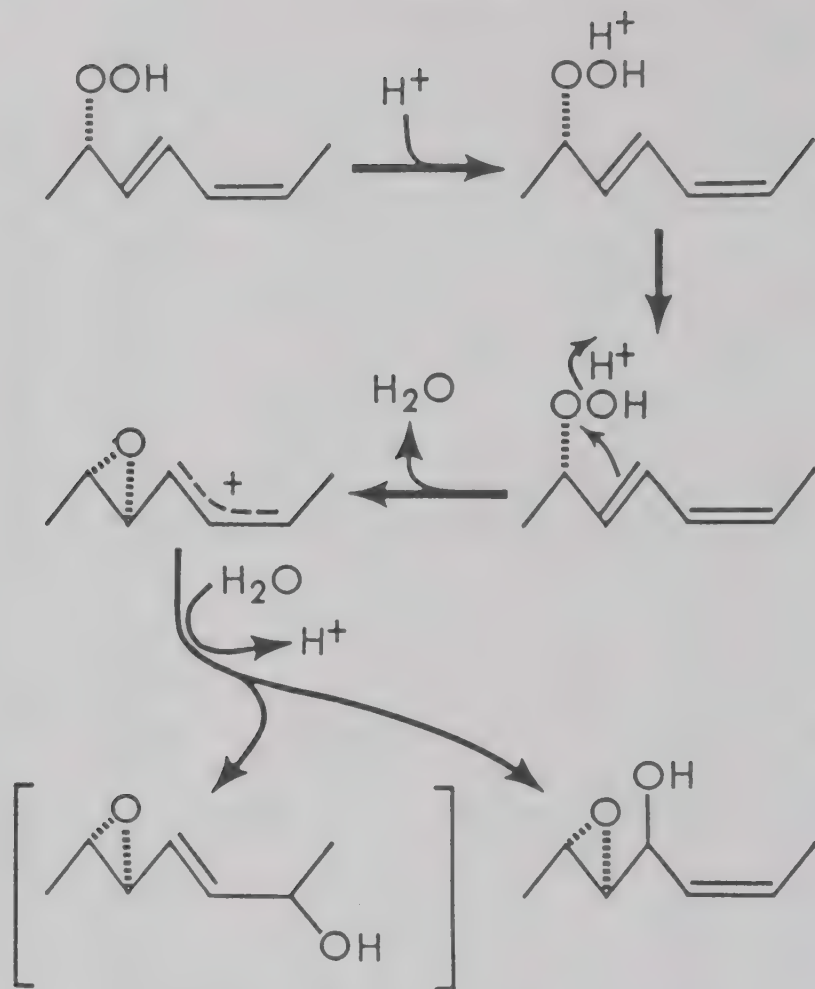


FIG. 9.12. Heterolytic formation of epoxyhydroxy fatty acids from [13S]-hydroperoxylinoleic acid in aqueous acid solutions. Structures are abbreviated to show only C-8 through C-14. The bracket indicates that this epoxide is very labile to solvolysis.

shown that epoxyhydroxy and trihydroxy fatty acids can be formed via heterolytic rearrangement of hydroperoxides. Although fatty acid hydroperoxides are cleaved by a Lewis acid in aprotic solvent as discussed above, protic acids (e.g., H_2SO_4) in protic solvent (H_2O) divert the course of reaction to the production of chiefly epoxyhydroxy fatty acids (Fig. 9.12) and their hydrolysis products, trihydroxy fatty acids (Gardner *et al.* 1984B). It is of interest to note the comparative difference in mechanisms between the protic and aprotic reactions (Figs. 9.11 and 9.12). The heterolytic nature of epoxide formation was ascer-

tained by the dependence of the reaction rate on the acid concentration and by substitution of protic solvent in the end products (Gardner *et al.* 1984A). At this time, it cannot be determined if the biological mechanism is either heterolytic or homolytic. Although soybean LOX (Garssen *et al.* 1976) and a rat lung preparation (Pace-Asciak *et al.* 1983) catalyzed the conversion of $^{18}\text{O}_2$ -labeled hydroperoxides into epoxyhydroxy fatty acids with retention of both hydroperoxide oxygens, it is not possible to ascertain whether the ^{18}O -labeled hydroxyl group originated from a "cage" transfer of H_2O (heterolytic) or hydroxyl radical (homolytic).

Hydroperoxide Isomerase. Zimmerman (1966) discovered that hydroperoxide isomerase from flaxseed converted the 13-hydroperoxide of linoleic acid into an α -ketol, 13-hydroxy-12-oxo-*cis*-9-octadecenoic acid. Subsequently, it was found that isomerase also formed a minor product from the 13-hydroperoxide, which was identified as the γ -ketol, 9-hydroxy-12-oxo-*trans*-10-octadecenoic acid (Gardner 1970). A number of workers (see reviews: Veldink *et al.* 1977; Gardner 1980) demonstrated that isomerase transferred one 13-hydroperoxide oxygen to the 12-oxo moiety, and the 13-hydroxy (α -ketol) or 9-hydroxy (γ -ketol) originated from H_2O . Based on these results and the conversion of [9S]-hydroperoxide to [9R]-hydroxyl in the α -ketol, Gardner (1979) proposed an epoxyallylic cation intermediate (Fig. 9.13). A further hydride transfer from an epoxide carbon to a vicinal carbon and substitution by H_2O completes the transformation. Although the proposed epoxyallylic cation intermediate is identical to that proposed for the acid-catalyzed reaction of hydroperoxides (Fig. 9.12), α - and γ -ketols are not obtained by acid treatment. However, acid catalysis of the 13-hydroperoxide of linoleic acid gives rise to the δ -ketols, 9-hydroxy-13-oxo-*trans*-10-octadecenoic and 13-hydroxy-9-oxo-*trans*-11-octadecenoic acids (Gardner *et al.* 1984B; Fig. 9.8).

Hydroperoxide Cyclase. Phytodienoic acid, 8[2(*cis*-pent-2'-enyl)-3-oxo-*cis*-cyclopent-4-enyl]octanoic acid, was biosynthesized from the 13-hydroperoxide of linolenic acid by the flaxseed enzyme, hydroperoxide cyclase (Vick and Zimmerman 1979A). This enzyme has been identified in a number of other plant species (Vick and Zimmerman 1979B). Vick *et al.* (1980) proposed a mechanism for the formation of phytodienoic acid that resembled the one postulated for hydroperoxide isomerase action (Fig. 9.14). Inasmuch as the mechanisms for isomerase and cyclase appear to be similar and the two enzymes are inseparable by chromatography, it has been suggested that they are the same

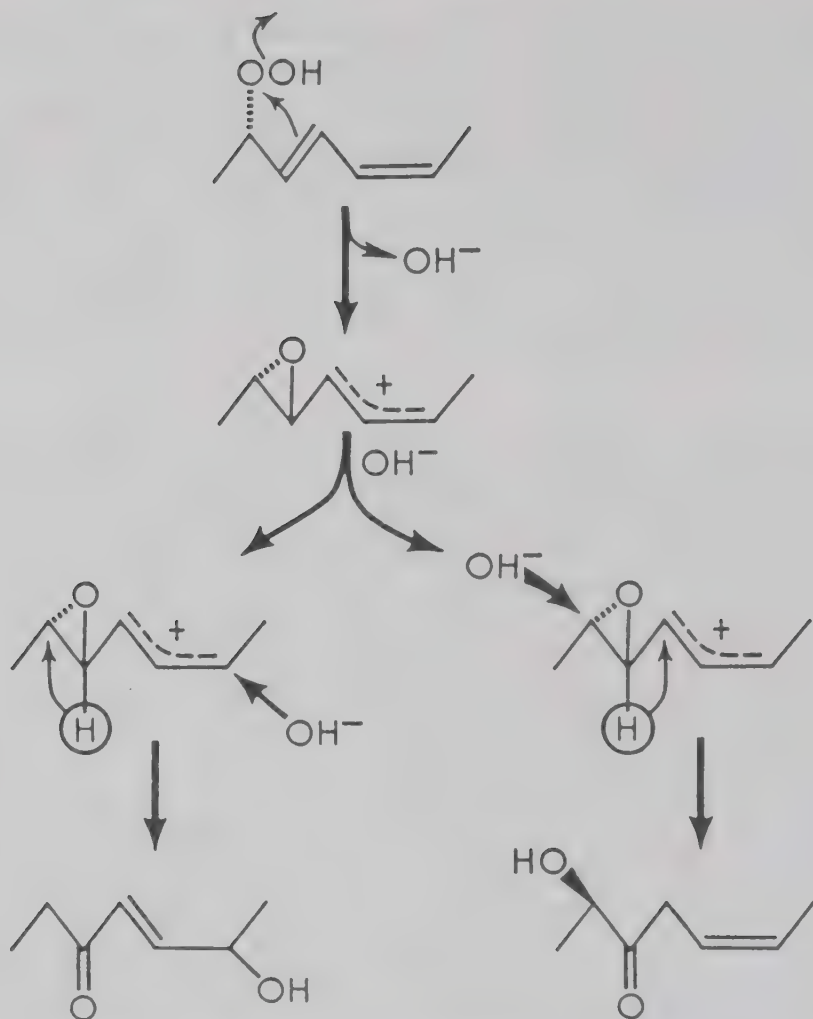


FIG. 9.13. Heterolytic mechanism of hydroperoxide isomerase action on [13S]-hydroperoxylinoleic acid. Structures are abbreviated to show only C-8 through C-14.

enzyme (Vick and Zimmerman 1981). Phytodienoic acid has not yet been obtained by a chemical reaction of linolenic acid hydroperoxide.

SIGNIFICANCE OF THE CASCADE

The arachidonic acid cascade is immensely important to the physiology of animals, and new discoveries probably will be forthcoming. In addition to the reviews by Nelson *et al.* (1982) and Marcus (1978), one should consult other numerous reviews available in this field. On

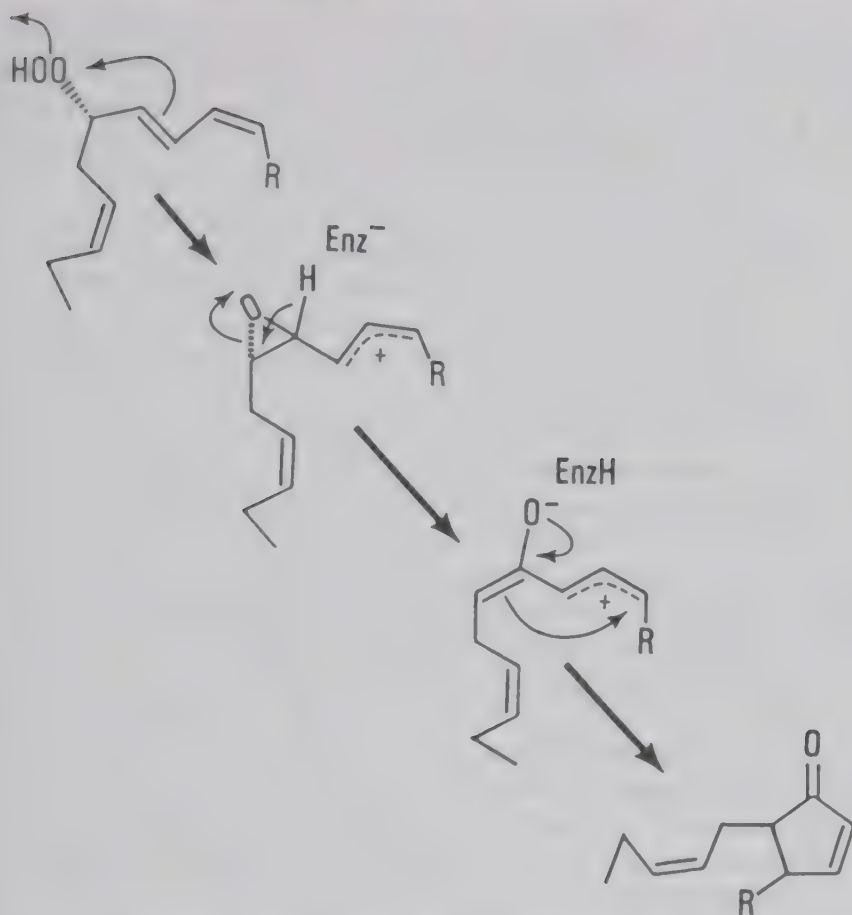


FIG. 9.14. Heterolytic mechanism of hydroperoxide cyclase action on [13S]-hydroperoxylinolenic acid. $R = \text{---}(\text{CH}_2)_7\text{CO}_2\text{H}$.

From Vick et al. (1980).

the other hand, the significance of the linoleic/linolenic acid cascade to plant physiology is only beginning to be understood, thus this area of investigation will be reviewed in detail. Certain aspects of physiological significance have been sparsely studied, and, as a result, some of the hypotheses discussed are not firmly established. It is hoped that this review will stimulate further work with the cascade in plants.

Localization of Plant Cascade Enzymes

To understand the physiological role of the cascade, it should be recognized that these enzymes are highly organized within the plant cell. The cascade enzymes and other lipid-active enzymes are largely localized in specific subcellular organelles or within organelle membranes. Seed germination is known to stimulate lipase activity. In

soybean seedlings, lipase is located within the glyoxysomes, and the triglyceride-containing spherosomes contain a monoglyceride lipase (Lin *et al.* 1982). In corn seedlings, lipase resides wholly within the membranes of the spherosomes (Lin *et al.* 1983). Still other oilseeds develop lipase-containing appendages emanating from the spherosome (Wanner and Theimer 1978). In germinating seeds, the lipase releases fatty acid from triglyceride mainly for fatty acid utilization by glyoxysomes. Glyoxysomes β -oxidize fatty acids to acetyl coenzyme A, which is subsequently utilized for carbohydrate synthesis (Tolbert 1981). The synergism of spherosomes and glyoxysomes is indicated by their close proximity during fat utilization (Wanner and Theimer 1978). Although the cascade enzyme, LOX, usually increases in activity during seed germination, it is not known if the lipase-released fatty acids are available to this enzyme. And, it is not known what role LOX has in the germination process. In corn seedlings, both LOX and the hydroperoxide-decomposing enzymes increase during germination (Vick and Zimmerman 1982) in parallel to the increase in lipase activity (Lin *et al.* 1982). Other oilseeds display similar patterns of LOX and hydroperoxide-decomposing activities during germination (Vick and Zimmerman 1981, 1982). In soybeans, LOX has been localized microscopically by immunofluorescence staining with anti-LOX immunoglobulin G (Vernooy-Gerritsen *et al.* 1983). Using this technique, these investigators demonstrated that LOX in the initial phase of soybean germination was found distributed throughout the cytoplasm of cotyledon cells; but after 2 days, the enzyme was located only in the epidermis layer. A few days later, the hypodermis, epidermis, and vascular bundle sheaths contained LOX. Following an initial absence of the enzyme in newly developed leaf tissue, LOX eventually developed throughout the leaf cell, except for the nucleus and vacuoles. As the soybean leaf matured, LOX became associated only with the chloroplasts. Others have also noted the presence of LOX in chloroplasts (Douillard and Bergeron 1981; Haydar and Hadziyev 1973), particularly in etioloplasts (Haydar and Hadziyev (1973) or chloroplasts from young leaves (Douillard and Bergeron 1981). Hydroperoxide lyase, which utilizes the products of LOX, also is often located in chloroplasts (Wardale *et al.* 1978; Hatanaka *et al.* 1982). The pattern of distribution of LOX, in particular, is suggestive of a regulatory function for this enzyme.

Role of Plant Cascade Enzymes in Wound Defense and Senescence

The wounding of plant tissue causes an increase in the levels of certain end products of the linoleic/linolenic acid cascade (Vick and

Zimmerman 1982). Traumatol, a wound hormone similar in structure to traumatin, is a direct product of the cascade, and it originates from hydroperoxide lyase cleavage of the 13-hydroperoxide of linoleic or linolenic acid (Zimmerman and Coudron 1979). Other products of hydroperoxide lyase action, such as hexenal and hexanal, may also function in plant protection. Apparently, plants respond to a "message" from insect-damaged neighbors and thereby increase their defense mechanisms (Schultz 1983). Ethylene was suggested as the message carrier, but volatile aldehydes, like hexenal and hexanal, possibly could function in this capacity. It is noted that the enzymes responsible for hexenal/hexanal biosynthesis (LOX and hydroperoxide lyase) are localized in leaves (Sekiya *et al.* 1983) and become activated when leaves are damaged. Hexenal (Major *et al.* 1960) and LOX in the presence of substrate (Senser and Grosch 1975) are potent inhibitors of fungal growth. The inhibition by LOX may be due to the primary product of LOX (hydroperoxides) or secondary products of hydroperoxide degradation. Both 9- and 13-hydroxyoctadecatrienoic acids, probably originating from LOX oxidation of linolenic acid, were formed by rice leaves and prevented the growth of pathogenic rice fungi (Shimura *et al.* 1983).

The cascade enzymes also may contribute to plant senescence. The senescence hormone, jasmonic acid, is biosynthesized from phytodi-enoic acid (Fig. 9.15), a product of hydroperoxide cyclase action (Vick and Zimmerman 1983). The biosynthesis of jasmonic acid is probably the best documented example of a physiological role for the linoleic/linolenic acid cascade in plants. Xanthoxin, which also promotes senescence, has been produced *in vitro* by cooxidation of the carotenoid, violaxanthin, during the oxidation of linoleic acid by LOX (Firn and Friend 1972). ^{14}C -Labeled xanthoxin was transformed by shoots of tomato and dwarf bean into the structurally related phytohormone, abscisic acid (Taylor and Burden 1973). In plants, abscisic acid functions in a number of important ways, such as triggering abscission, counteracting the effects of water stress, and causing senescence. Although the investigations cited above seem to point to a violaxanthin precursor for abscisic acid biosynthesis, compelling evidence from a number of laboratories indicated that abscisic acid is biosynthesized *de novo* from mevalonate pyrophosphate (Milborrow 1974). It would appear, however, that certain aspects of abscisic acid biosynthesis are not fully resolved at this time. For example, it is difficult to explain the ^{18}O -labeled carboxylic acid group of abscisic acid obtained from leaves exposed to $^{18}\text{O}_2$ (Creelman and Zeevaart 1984).

Ethylene causes seed sprouting, flowering, and fruit ripening, as well as other physiological effects on plants. For many years LOX was either directly implicated in ethylene biosynthesis or thought to

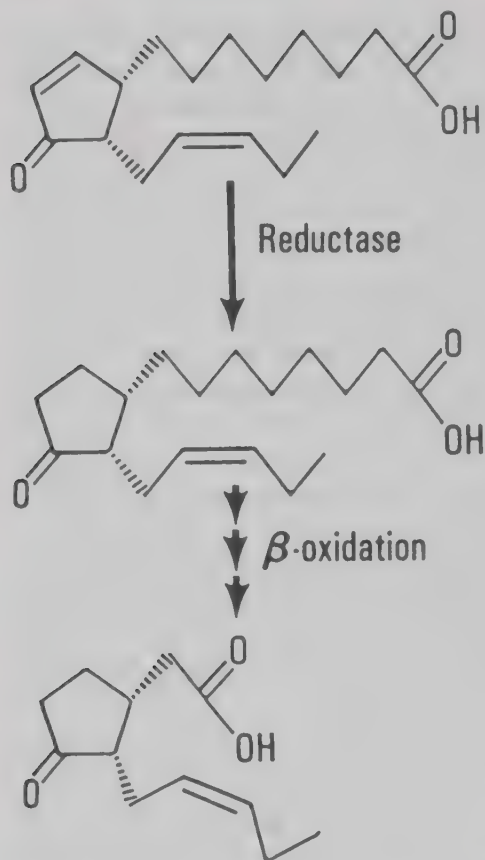


FIG. 9.15. Biosynthesis of jasmonic acid from phytodienoic acid.

Adapted from Vick and Zimmerman (1983).

play a secondary role (Eskin *et al.* 1977). Recent work showed that ethylene was biosynthesized from methionine via aminocyclopropane-carboxylic acid (ACC) (Adams and Yang 1979). The key unresolved question about ethylene biosynthesis involves the O_2 -requiring conversion of ACC to ethylene, which is postulated to be a free radical process. Pirrung (1983) modeled ethylene biosynthesis by electrochemical oxidation of ACC. According to him, the reaction was characterized by a two-stage single-electron oxidation of the amino group of ACC, each of which was followed by β -scission of the radicals formed by the two oxidations. It seems plausible that LOX-produced hydroperoxides could supply the oxidizing equivalents needed for the conversion of ACC to ethylene. For example, ethylene was formed from ACC in the presence of pea microsomes, provided that both LOX and linoleic acid were supplied (Legge and Thompson 1983). These latter workers assumed that the active agent was O_2^- (superoxide); however, O_2^- is a very weak oxidant. It seems much more likely that either peroxy or oxy radicals from the hydroperoxides were abstract-

ing the amino hydrogens of ACC for the radical conversion to ethylene. Thus, H abstraction possibly could carry out the oxidation observed electrochemically by Pirrung (1983). It is noted that LOX activity in fruit increases considerably just prior to fruit ripening and the onset of ethylene production (Meigh *et al.* 1967).

The application of certain growth-promoting hormones, like kinetin and gibberellic acid, has been claimed to reverse the effects of senescence-related accumulation of lipid peroxides in leaf segments (Dhindsa *et al.* 1982). Application of cytokinin to pea plants caused considerable reduction in endogenous LOX levels (Grossman and Leshem 1978), lending further credence to a hypothesis that LOX is involved in senescence.

Possible Control Functions of Cascade Enzymes

The function of cascade enzymes, particularly LOX, in the control of metabolic functions of the plant has been hypothesized only recently. According to Douillard (1980), LOX in the chloroplast may regulate the enzymes involved with carbon fixation, particularly in young chloroplasts which are not yet photosynthesizing. Active cycling of the Calvin cycle and the C-4 CO₂ fixation pathway requires reducing equivalents supplied by ferredoxin-reduced thioredoxin to maintain the thiol-active form of key enzymes, namely, fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, NADP-glyceraldehyde-3-phosphate dehydrogenase, phosphoribulokinase, and NADP-malate dehydrogenase (Buchanan *et al.* 1979). According to Douillard's hypothesis (1980), LOX supplies oxidizing equivalents in the form of hydroperoxides to convert the thiol enzymes to their inactive disulfide forms, thereby terminating the normal functioning of photosynthetic carbon fixation. Conversely, hydroperoxides would activate the oxidative pentose phosphate cycle by converting glucose-6-phosphate dehydrogenase into the active disulfide form.

The function of LOX in plant mitochondria is currently a controversial area of research. Although mitochondria do not appear to contain LOX *per se*, it is difficult, if not impossible, to remove 100% of the enzyme (Siedow and Girvin 1980; Shingles *et al.* 1982; Dupont 1981). The controversy has centered on the so-called "alternate respiration" of mitochondria, which is the respiration caused by a cyanide-insensitive oxidase that is sensitive to inhibition by propyl galate and salicylhydroxamic acid. It has been suggested that alternate respiration is actually due to LOX activity (Parrish and Leopold 1978), but this assertion has been contested by others. For example, potato slice mitochondria has an alternate respiration that could not be as-

cribed to LOX activity (Shingles *et al.* 1982). On the other hand, alternate respiration disappeared from wheat mitochondria after LOX activity was lost during mitochondrial purification (Goldstein *et al.* 1981). Reportedly, the respiration of soybeans during the first hours of imbibition displayed alternate respiration (Yentur and Leopold 1976). Siedow and Girvin (1980) found this respiration was cyanide sensitive, and thus was not alternate respiration. The confusion in this area of research possibly has been explained by recent work. According to Dupont *et al.* (1982), fatty acid hydroperoxides were responsible for causing alternate respiration. These latter workers (Rustin *et al.* 1983) hypothesized that alternate respiration is not due to a new terminal oxidase, but is caused by direct oxidation of mitochondrial ubiquinone by fatty acid peroxy radicals. It is interesting to note that mammalian mitochondria are also affected by fatty or organic hydroperoxides. Exposure of *t*-butylhydroperoxide to rat liver mitochondria caused sequential events, each dependent on the preceding event: (a) NAD(P)H oxidation; (b) Ca^{2+} release; and (c) mitochondrial swelling (Moore *et al.* 1983). With rat heart mitochondria, linoleic acid hydroperoxide stimulated state four respiration, uncoupled oxidative phosphorylation, caused swelling, and released malate dehydrogenase (Shiotani *et al.* 1980). Methyl hydroperoxyepoxyoctadecenoate similarly affected both rat heart and liver mitochondria at lower levels (Imagawa *et al.* 1982). The latter results were attributed to uncoupling of oxidative phosphorylation (Imagawa *et al.* 1982) and inhibition of both NADH-oxidase and NADH-ubiquinone reductase (Imagawa *et al.* 1983). This latter conclusion should be compared to the theory that alternate respiration in plant mitochondria is owing to ubiquinone oxidation by hydroperoxides (Rustin *et al.* 1983).

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Oxidation-Induced Changes in Foods

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INTRODUCTION

A frequent cause of poor shelf life and off-flavors in processed foods is oxidation of lipids, primarily the unsaturated fatty acids of storage oil and membrane lipids in plant foods, and the saturated fats in animal products and cooking fats used for cooking/processing both plant and animal foods. Even during storage, fats can undergo oxidative changes that give rise to objectionable flavors and odors. In general, the higher the unsaturation of the fat, the greater the susceptibility to oxidation. Oxidation of fats proceeds via a free radical mechanism in three basic steps: initiation (addition of oxygen to the fat), propagation (generation of hydroperoxides and free radicals), and termination (combination of free radicals with each other or with other compounds, the formation of further peroxides and the release of oxygen back to the system). Hydroperoxides are the major products of lipid oxidation. They can break down to secondary products (aldehydes, al-

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cohols, ketones, acids), or they can react with proteins and enzymes in the food products. Oxidized lipid free radicals, which were studied for scientific interest only a few years ago, have stimulated considerable interest by food scientists and clinicians because of increasing evidence for their association with toxic or carcinogenic agents. Our diet today contains several chemicals that are mutagenic or carcinogenic (Abelson 1983; Ames 1983), some formed during cooking via reactions involving fats or proteins. The influence of diet on rate of formation of certain chemically induced and spontaneous tumors has been well documented (Black 1983). Rancid fats are sometimes implicated in these disorders, but the pungent odor of rancid foods will usually deter their consumption.

Although the physiological effects of peroxidized lipids may be getting more publicity, there still is considerable research on causes of lipid oxidation in foods and the effects on quality and shelf life. This problem is not confined to oilseeds and animal products, but is also of concern in fresh and processed foods derived from legumes, pulses, cereals, and some vegetables. In addition to lipids, oxidation of polyphenolic pigments can also give rise to off-flavors, undesirable colors, and changes in texture/functional properties due to cross-linking of macromolecules during processing and/or storage. The volatile products of lipid oxidation and autoxidation processes in foods have been reviewed by others (Frankel 1982; Simic and Karel 1980) because of their significance in flavor/aroma/quality of virtually all processed foods. Some aspects of this broad subject are covered by others in this volume. This report concentrates on three examples of oxidation-induced changes and their effects on quality in an oilseed, on deterioration of frying fats used in fast food services, and on alteration of cooking properties of aged rice.

LIPID OXIDATION IN PROCESSED PEANUTS

Peanuts (groundnuts) are a major oilseed crop grown in tropical/subtropical countries. The United States crop in 1983 was estimated at 1.55 million tons (Anon. 1983A). Peanuts contain 50–55% oil and 27–30% protein in close proximity to each other. Under normal conditions, no adverse changes take place, but handling steps prior to processing (e.g., drying, shelling, blanching to remove skins, long-term storage, shipping) can activate lipoxygenase and other undesirable enzymes in raw peanuts, which ultimately lower quality of the final product (St. Angelo *et al.* 1979; Shewfelt and Young 1977). Since most of the U.S. crop is processed into edible products such as peanut butter, candy, cookies, confections, and salted nuts rather than

being crushed for the edible oil, processing and storage conditions can introduce nonenzymatic oxidation of the fatty acids which will then be incorporated into the finished product.

Peanuts contain both storage and functional lipids. Storage lipids consist primarily of triglycerols that serve as sources of energy to be mobilized upon demand by the seed. Functional lipids consist of phospho-, glyco-, and sulfolipids, sterols and their derivatives, etc., that function in membranes, subcellular organelles, or other specialized particles. Oxidation of storage lipids is a major source of off-flavors and decreased quality in processed peanuts. If raw peanuts are handled carefully and stored under optimum conditions, they can be stored for up to 5 yr before being processed (Shewfelt and Young 1977). If mishandled during digging, drying, shelling, and storage, they become completely inedible within a month.

Lipoxygenase is the principal catalyst of fatty acid oxidation in raw peanuts (St. Angelo *et al.* 1977, 1979). It can be activated by damage to the raw seeds that ruptures cell walls and places enzyme and substrate in contact with each other. The longer the storage period, the greater will be the extent of enzyme-induced deterioration. If enzyme activity is excessive, rancid odors may be so pronounced as to preclude any use of the peanuts in edible products. In general, however, raw nuts will not develop the foul rancid odors that appear in roasted nuts of the same age. This may be due to the faster rate of nonenzymatic lipid peroxidation that develops in roasted nuts compared to enzymatic catalysis in raw nuts, such as peanuts during storage (Fig. 10.1). After 1 yr of storage at 4°C in sealed glass jars with air headspace, roasted peanuts developed lipid peroxides eight times faster than did raw peanuts (St. Angelo and Ory 1975A).

Lipid-Protein Interactions

Though not visibly evident, there is also considerable binding of lipid peroxides to functional groups of amino acids in proteins. The most susceptible reactive groups are the —SH (Cys), —OH (Ser, Thr, Tyr), —NH₂ (Lys, Arg), —COOH (Glu, Asp), —NH (His), and —SCH₃ (Met). Lipid-protein interactions occur in both raw and roasted peanuts. As seen in Fig. 10.2, the electrophoretic disc gel patterns of proteins stained for lipid-protein complexes in raw and roasted peanuts are slightly different; two bands in raw and three in roasted peanuts. One protein that forms a lipid-protein complex in raw peanuts may be broken into more than one band in roasted samples. The lipid-stained gels indicate greater binding affinity of peroxidized lipids to arachin and conarachin, the large globulins, than to smaller proteins (St. Angelo and Ory 1975A). Peanuts that are processed for peanut

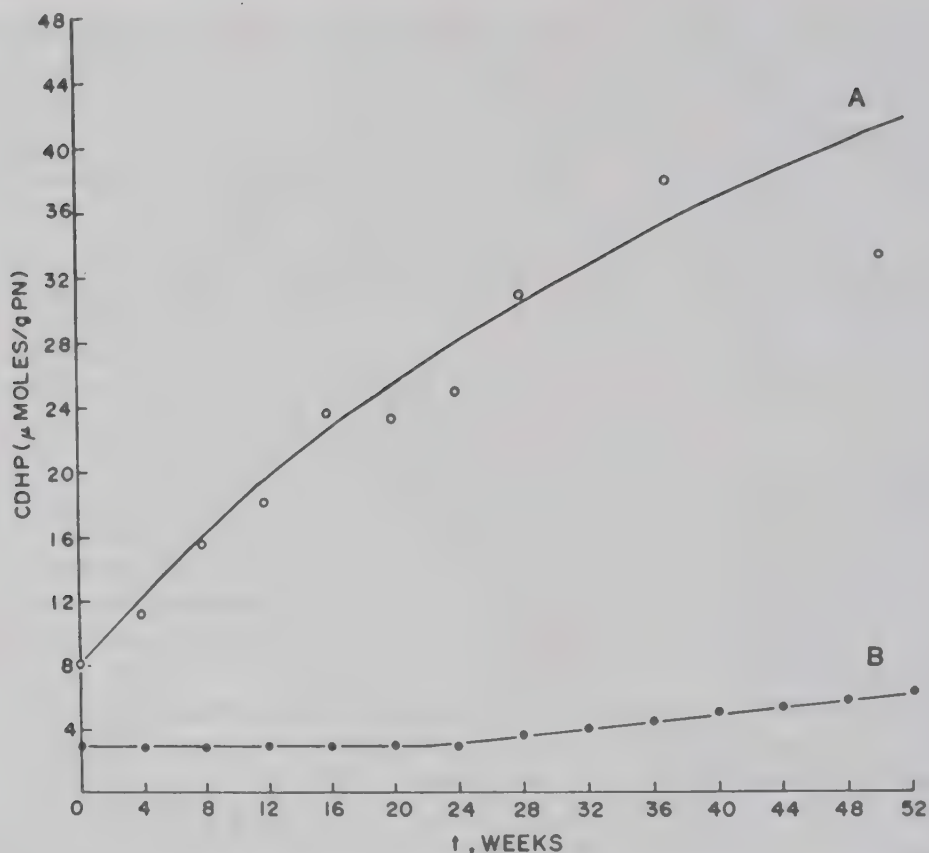


FIG. 10.1. Rates of formation of conjugated diene hydroperoxides in roasted (A) and raw (B) peanuts during storage at 4°C in sealed glass jars for 1 yr. Each point is average of 12 samples.

From St. Angelo and Ory (1975A).

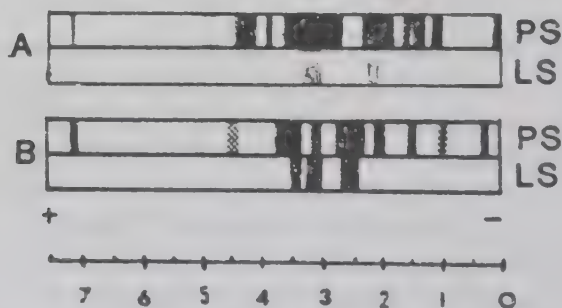


FIG. 10.2. Gel electrophoretic patterns of proteins extracted from raw and roasted peanuts. Fresh raw peanuts (A) and roasted peanuts (B) were stained for both protein, by Coomassie Blue (PS), and lipid-protein complexes, by Oil Red O (LS). Migration is toward the anode.

TABLE 10.1 PROTEIN SOLUBILITY IN 10% NaCl AFTER ROASTING

Sample	CDHP units ^a	Protein in residue (%)	Soluble protein (%)	Total recovery (%)
Peanut butter	7.27	53.6	13.8	67.4
Peanut butter	11.38	53.3	15.5	68.8
Whole peanuts	6.54	61.2	17.2	78.4
Whole peanuts	38.93	55.8	17.1	72.9

^a Millimoles of conjugated diene hydroperoxide/gram of peanut butter or peanuts.

butter manufacture, salted nuts, or candies are roasted at high temperatures for specific times, depending upon the size of the seeds. Heat denatures most proteins, but apparently does not dissociate the lipid-protein complexes. This also suggests that roasted peanuts not properly packaged to retard oxidation may undergo lipid peroxidation with subsequent lipid-protein interactions during storage. This is particularly important in homogenized products like peanut butter and cookies or candies that incorporate peanut butter in the formulation.

In addition to lowering bioavailability of amino acids like lysine, binding of lipid peroxides to proteins may affect protein solubility of roasted peanuts. As peroxidation increased in peanut butter and roasted peanuts during storage, protein solubility seemed to rise slightly in peanut butter but not in whole peanuts (Table 10.1). Salt extraction of the oil-free meal left larger amounts of insoluble protein in the residue, but solubilized some protein. The percentage of residue and total protein recovered from whole roasted peanuts decreased, but the samples were not statistically analyzed because of the small numbers assayed.

Protein Fluorescence

Conformation of proteins in roasted peanuts is also altered as a result of the binding with lipid peroxides. This can be determined by measuring fluorescence spectra of the proteins at 401–404 nm. Conjugated Schiff bases formed between malonaldehyde and amino acids fluoresce at 401–404 nm (Chio and Tappel 1969). NaCl-soluble proteins extracted from fresh and 1 yr-stored roasted peanuts were scanned at 400 nm, the absorption maximum for these peanut proteins (St. Angelo and Ory 1975B). Fluorescence spectra of the salt-soluble proteins extracted from rancid peanuts, both raw and roasted, were depressed when compared to those of fresh peanuts. The reason for this is unknown, but the apparent quenching by lipid peroxides in roasted peanut proteins may result from changes in molecular bonding.

Storage Effects

As mentioned earlier, handling and storage conditions will affect quality of peanuts before processing. Marzke *et al.* (1976) showed that peanuts stored in-shell resisted damage better than shelled peanuts. Peanuts were stored in atmospheres of nitrogen, carbon dioxide, or air, but storage temperature (4.5°C vs 26.6°C) affected quality more than atmosphere. At 4.5°C, peanuts stored in carbon dioxide or nitrogen for 1 yr developed less skin discoloration, staleness, or rancidity than those stored in air.

Stability of macademia nuts was affected by quality of the roasting oil (Cavaletto and Yamamoto 1971). In commercial processing, macademia nuts are roasted in coconut oil (saturated fat) containing antioxidants to extend usable life of the oil and retard rancidity development in the nuts. Free fatty acids, color, and viscosity of the oil increased during 13 weeks of roasting use, and antioxidant loss in the roasting oil was rapid. Nuts treated with antioxidants were more stable than untreated nuts, regardless of the level of vacuum packaging, but vacuum packaging increased stability of untreated nuts.

The limited tests described for peanuts should be applicable to other oil-containing nuts that are roasted during processing, such as macademia nuts, cashews, pecans, almonds, hazelnuts, and sunflower seeds. Lipid peroxides can affect flavor, odor, protein solubility and conformation, nutritional value, and in some cases, color of the processed product. Where binding of lipid peroxides is to lysine, this could affect nutritional quality, since lysine is already low in oilseeds.

QUALITY OF FRYING FATS FOR FAST FOOD SERVICES

One of the fastest growing industries today is that of food services. More Americans are eating out today than ever before. In 1960, 26% of the total food dollar was spent on food services. By 1981, this increased to 38% (Anon. 1983B). The dollar value of franchise restaurant sales increased from \$30 billion in 1981 to \$38.4 billion in 1983. Over 10% of these sales consisted of deep-fried chicken parts. In addition to chicken, large quantities of French fried potatoes, onion rings, fish fillets, and other products are deep fried during processing. This requires enormous quantities of cooking fats that must resist high temperatures for long periods without losing quality, since acceptance of the fried product will be adversely affected.

Both deep fat and pan frying are used by restaurants, but the fast

food services employ primarily deep fat frying because the foods are well prepared, are crisp on the outside but tender on the inside, are more flavorful, have an appealing aroma, and deep fat is a faster, more efficient heat-transfer medium than dry oven heat or boiling in water. Frying temperatures range from 182° to 191°C and, depending upon the type of cooking fat and the numbers and sizes of pieces to be fried, the fat may be used for 10–14 days. Frying fats deteriorate at different rates when heated. The five stages of progressive oxidative deterioration of frying fats involve an induction period, followed by peroxide formation, decomposition, polymerization, and degradation. During deep-fat frying, vegetable oils can undergo oxidation, cyclization, polymerization, degradation to volatile compounds, and hydrolysis. It is the combination of these chemical changes that causes off-flavors, rancid aromas, greasy mouth feel, and impaired nutritional value in fried foods. Maintaining the quality of frying fats under severe processing conditions is, therefore, critical to acceptance of the foods fried in them.

Deterioration of Frying Fats

In fast food restaurants, frying fats are subjected to severe conditions: high temperatures for 10–12 hr/day for 10- to 14-day periods, generally in uncovered deep fryers. The combination of heat, air, light, moisture, and fragments from foods being fried contributes to oxidation of the fats, which may be pure vegetable oil, partially hydrogenated vegetable fat, or blends of vegetable and animal fats. Hydrolysis of a cooking fat can occur when moist foods (e.g., battered chicken or fish portions) are fried in the hot fat. Steam derived from this moisture can cause hydrolysis of the fat to fatty acids. On the other hand, this steam can also act as a form of steam distillation to remove off-flavors and odors derived from low-molecular-weight carbonyls, alcohols, and acids as soon as they are formed. The volatiles still present in a frying fat can be measured by the direct gas chromatographic method of Dupuy *et al.* (1973) to indicate extent of deterioration of the fat. Dupuy and co-workers showed that as quality of the oil decreased, the concentration of volatiles increased. Deterioration of the frying fat depends upon four general factors: temperature of the fat, the degree of exposure of the fat to air, the amount of nonfat ingredients contaminating the fat, and the turnover rate of the fat. The useful life of frying fats has been a major problem for the fast foods industry. Since so many reactions can occur in the fat and so many variables must be considered during the frying operation, the decision of when to discard a frying fat is most difficult. A series of experi-

ments was designed to examine the quality of frying fats under severe conditions encountered in fast food services and to try to retard or inhibit deterioration.

Ascorbyl Palmitate Addition

To simulate such conditions, an all vegetable-soybean oil (V-S) and an animal-vegetable blended shortening (A-V) were heated at 185°C for 10–11 hr/day for a 10-day period in open 400-ml beakers, 200 g/beaker, with and without 0.02% ascorbyl palmitate (AP), a generally recognized as safe (GRAS) foodgrade antioxidant permitted in unlimited levels under Title 21, 1982 Code of Federal Regulations: 182.3149. AP was found to be more effective than either BHA or BHT in preventing oxidation of vegetable oils (Cort 1982). Ingestion of AP poses no health hazard for consumers, since metabolic breakdown yields ascorbic acid and palmitic acid. Addition of 0.02% AP reduced brown color development in both fats compared to the controls without AP. Darkening of the brown color was reduced 25–30% with AP added at the beginning of the heating period.

AP also reduced peroxide formation in both the saturated (A-V) and unsaturated (V-S) cooking fats when it was added at the beginning and every other day of the tests for 10 days (Figs. 10.3 and 10.4). In both cases, AP exerted a beneficial effect by reducing peroxidation of the cooking fats. Peroxide formation increased normally up to 4 days of frying use, then between 4 and 6 days the buildup of peroxides seemed to diminish somewhat, increasing again by 8 days of frying. This suggests that lipid peroxides may be breaking down to form secondary products. Since AP is an oxygen scavenger, a reduction in peroxides might be expected if fresh AP is added every other day. In comparing series I (single addition of AP) and series II (additions every other day), it was found that peroxide values developed more slowly in series II, as might be expected. A single addition of AP to the shortening (A-V FAT + AP) did not seem to reduce peroxide values as it did in vegetable oil (V-S OIL + AP) or as it did for both frying fats with multiple additions of AP in series II.

Since the reduction in rate of peroxide value formation at 4–6 days of frying suggested a breakdown of peroxides to secondary products, the fats were analyzed for volatiles profiles by the rapid gas chromatographic method of Dupuy *et al.* (1973). Results of these analyses confirmed the indications of fat deterioration suggested by the peroxide values. The total volatiles for both the V-S and the A-V fats increased steadily over 8 days of heating, then seemed to decrease between 8 and 10 days, possibly because of increased polymerization

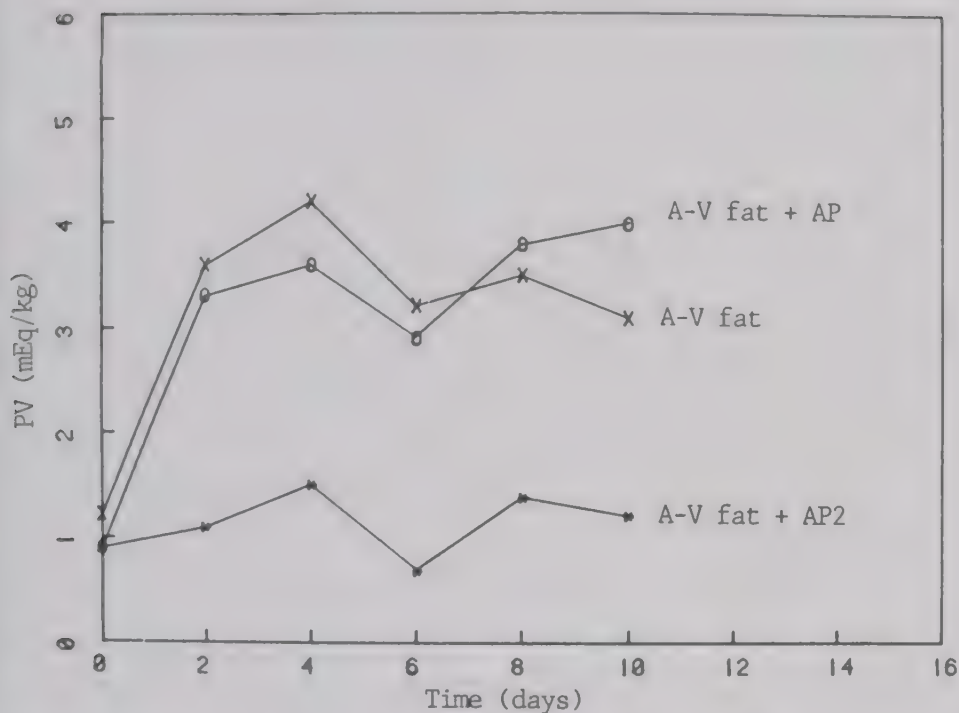


FIG. 10.3. Peroxide values of animal-vegetable shortening with and without ascorbyl palmitate (AP) during 10 days of cooking at 185°–188°C. Cooking time was 10–11 hr/day; A-V, animal-vegetable shortening without antioxidant; A-V FAT + AP, with an initial dose only of 0.02% AP; A-V FAT + AP2, with 0.02% doses of AP at 0, 2, 4, 6, and 8 days during cooking.

and/or cyclization of the peroxides and volatile products. These secondary volatile products contribute to the off-flavors and odors of oxidized fatty foods. Addition of AP to the unsaturated vegetable oil (V-S) produced significant decreases in formation of volatiles (Fig. 10.5). Results with the saturated animal-vegetable shortening (A-V) also showed increases in formation of volatiles with time of heating and decreases in volatiles with single or multiple additions of AP, but less than those exhibited by the V-S oil. V-S oil contains more polyunsaturated fatty acids, whereas A-V shortening contains more stearic and palmitic acids. Deterioration of frying fats and oils at high temperatures is complicated, since both oxidations and thermolytic reactions are taking place together. However, both saturated and unsaturated fatty acids undergo chemical decomposition when exposed to high heat, oxygen in air, light, etc. (Simic and Karel 1980). Deep-frying fats deteriorate when heated for long periods of time when exposed to air with or without cooking foods in them. Addition of AP can retard the

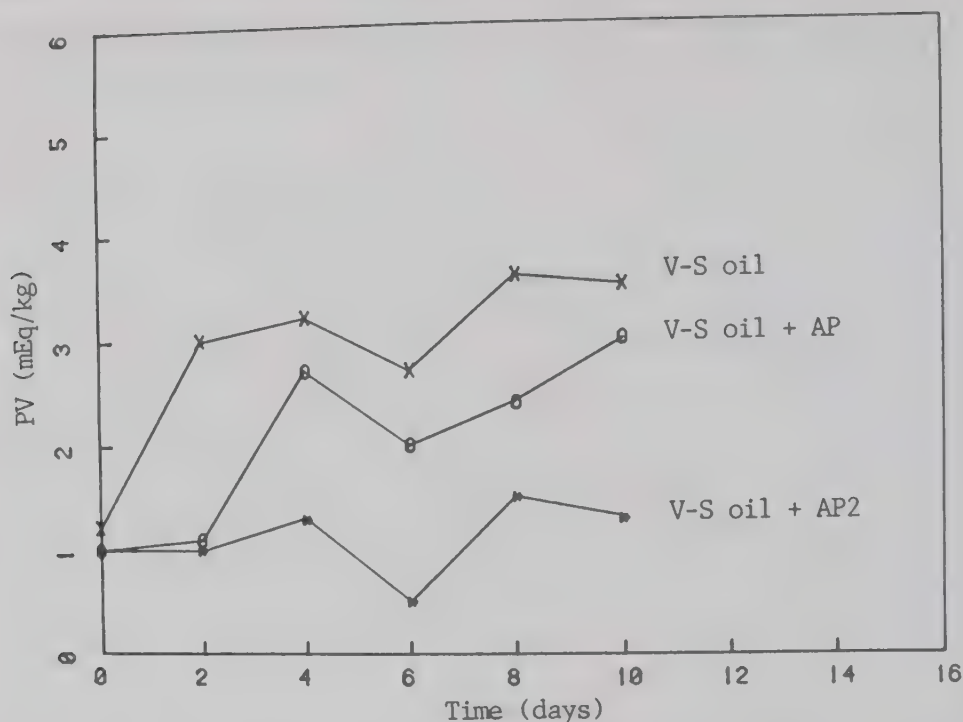


FIG. 10.4. Peroxide values of vegetable–soybean oil with and without ascorbyl palmitate (AP) during 10 days of cooking at 185°–188°C. Conditions same as in Fig. 10.3; V-S, vegetable–soybean oil without AP; V-S OIL + AP, with an initial dose of AP; V-S OIL + AP2, with doses of AP every other day as in Fig. 10.3.

effects of deterioration, i.e., brown discoloration, peroxide value, and total volatiles derived from degradation of fatty peroxides. These effects of AP suggest that this GRAS antioxidant should be an acceptable means of extending the useful life of frying fats. BHA, a GRAS antioxidant permitted at limited levels, was found to be more effective than BHT during static heating of vegetable oils (Augustin and Berry 1983).

OXIDATIVE CROSS-LINKING OF POLYPHENOLICS IN RICE

In cereal grains, cross-linking changes may be caused by oxidation of lipids (lipoxygenase action in whole grains/meals) or nonlipid components (polyphenolics). Cereal grains contain only 2–3% oil, primarily in the bran (aleurone) layer, so that in bran-free grains, such as milled rice, the principal causes of oxidative cross-linking changes

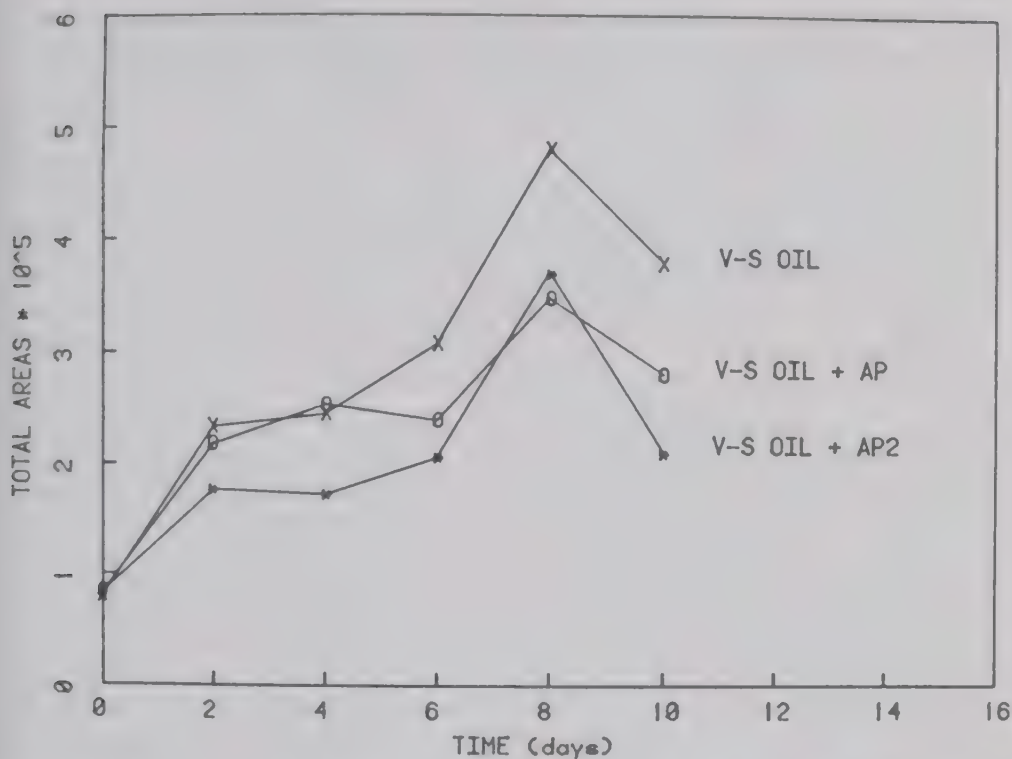


FIG. 10.5. Total volatiles of vegetable-soybean oil with and without ascorbyl palmitate (AP) during 10 days of cooking at 185°–188°C measured by direct gas chromatography (area of all GC peaks). Conditions same as in Fig. 10.4.

are generally the polyphenolic compounds. Covalent cross-linking of large polymers generally has a stabilizing effect on structure that can induce striking changes in properties of the polymer, particularly cooking properties of high starch-containing foods. This could affect the cooking properties of products, such as baby foods, that depend upon proper gelling/viscosity of milled rice flour. Neukom (1980) showed that wheat flour pentosans could undergo oxidative gelation with added oxidizing agents, without heating and cooling. This is in contrast to usual gel formation of polysaccharides like starch, pectin, and agar. No specific oxidizing agents are required; these can be hydrogen peroxide plus peroxidase, ferric chloride, iodine solution, potassium ferricyanide, or linoleic acid plus lipoxygenase.

Ferulic acid, a phenolic compound, is readily oxidized, and small amounts are associated with wheat pentosans (Neukom 1980) and rice hemicelluloses (Mod *et al.* 1981). Diferulic acid is formed by oxidative coupling of two ferulic acid residues to cause cross-linking of the po-

lysaccharides to which they are bound. This cross-linking can affect both physical properties and cooking properties of products like baby foods, certain puddings, and foods for patients with celiac disease that require specific thickening action.

Long- and medium-grain rices were milled to remove the bran layer. The milled rice was ground to a fine flour, and water-soluble hemicelluloses were isolated from the bran layer and the milled rice flour. Using thin-layer chromatography, Mod *et al.* (1981) found that ferulic acid was associated with the hemicellulose. To examine the effects of hemicellulose and ferulic acid on viscosity of cooked rice flour, intact rice flour (not extracted) and hemicellulose-free rice flour (extracted) were mixed with water, heated, and viscosities measured in a Brabender Amylograph-Viscograph. Hemicellulose-free rice flour had different viscosities than that of intact flour. Addition of water-soluble endosperm hemicellulose back to long-grain intact rice flour produced no viscosity changes compared to the control, but addition to medium-grain intact rice flour raised the peak viscosity. Adding back the ferulic acid-free hemicellulose to the control rice flour had no effect on amylograph viscosity curves. Addition of ferulic acid-hemicellulose complex isolated from the bran layer to intact rice flour lowered the entire heating and cooling viscosity curves significantly, suggesting that the ferulic acid-polysaccharide complex may be responsible for the reduction in viscosity of cooked rice flour. Whether this is due to differences in composition, molecular weight, degree of branching in hemicellulose, or to the amount of ferulic acid in the rice endosperm is not known with certainty, but a role for ferulic acid may be possible based upon results of the viscosity tests. Ferulic acid is present in rice flour, probably bound by ester linkage. Upon oxidation, ferulic acid may somehow generate active oxygen species that fragment the hemicellulose to decrease viscosity. Such reactions would affect cross-linking and may be partly responsible for the changes noted in rice during long-term storage and for altered viscosities of cooked rice flour products.

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Controlling Acyl Transfer Reactions of Hydrolases to Alter Food Constituents

*T. Richardson*¹

INTRODUCTION

The hydrolases represent a large and diverse group of enzymes that play major roles in food processing and in the storage life of foods. More than 1500 hydrolases have been characterized in detail (Enzyme Nomenclature Committee 1979). They act to hydrolyze a wide variety of substrate molecules ranging from the cleavage of a specific bond in a simple substrate to multiple bonds in a variety of macromolecules. Endogenous as well as exogenous (added during processing or associated with microorganisms in food) hydrolases can be key components in the conversion of raw materials and ingredients into food products. Their action on available substrates can alter the texture, flavor, odor, and color of food systems to yield desirable food products. On the other hand, insufficient, excessive, or unwanted hydrolase activities can lead to undesirable quality attributes, including food spoilage. The food scientist must control the action of hydrolytic and other enzymes in foods in order to produce traditional and new

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food products and to maximize the storage stabilities of foods. An understanding of hydrolases at a more mechanistic level can supply the food scientist with insights to better control hydrolase activities and to design better processes and products.

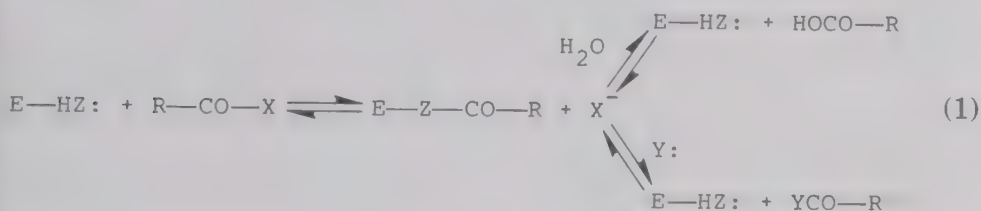
Although the molecular mechanism of action of hydrolases may differ, they catalyze the cleavage of susceptible bonds using the elements of water to effect bond scission. As suggested by Walsh (1979), it is convenient to consider the action of hydrolytic enzymes as involving a variety of group transfer reactions. In these group transfers, a limited number of functional groups are considered to be transferred first from the substrate to the enzyme, then to incoming nucleophiles. In the case of hydrolysis, the ultimate nucleophile is water or hydroxide ion. However, it is important to remember for the subsequent discussion that sufficiently high concentrations of other nucleophilic groups, e.g., amines or thiols, can compete effectively with water for the transferable group to yield the corresponding derivative. These enzyme-catalyzed reactions mainly involve the transfer of amino, phosphoryl, nucleotidyl, pyrophosphoryl, or acyl groups (Walsh 1979).

The acyl transfer reactions comprise a large portion of the hydrolase activities that are particularly relevant to food science. In general, enzymes acting on substrates containing ester and amide (peptide) linkages function via acyl transfer mechanisms. Thus, lipases, esterases, and proteases are representative groups of enzymes utilizing acyl transfer to alter substrates. This review will explore selected examples of acyl transfer reactions and their possible significance to changes during food processing and storage. Although the primary role of hydrolases is to cleave susceptible bonds, many of these enzymes have synthetic capabilities resulting from reversal of hydrolytic reactions. They are also capable of rearranging or restructuring complex substrates leading to products with properties different from the parent substrate. Acyl transfer reactions leading to synthesis or resynthesis of new bonds and resulting in different products will be emphasized.

ACYL TRANSFER

In acyl transfer reactions effected by enzymes, the acyl function of the substrate is ultimately transferred to water or to competing nucleophiles. However, the formation of enzyme-substrate intermediates and their subsequent reactions are crucial in determining the nature of the products. Often (but not always, as we shall see) a nucleophilic group in the active site of the enzyme is acylated to form a

covalent intermediate. As shown in reaction 1, water can attack this intermediate, leading to hydrolysis. Reversal of the hydrolytic reaction can lead to new condensation products. On the other hand, sufficiently high concentrations of other nucleophiles ($Y:$) can compete with water for the acylated intermediate, leading to the synthesis of new bonds.



The rates of the competing reactions will be affected by the relative concentrations of water and $Y:$. Obviously, as high a concentration of $Y:$ as is practicable is necessary for significant synthesis of new bonds. In addition to the concentrations of competing reactants, peculiarities of the enzyme involved in the reactions are important in determining the course of enzymatic events. For example, the proteolytic enzyme papain has been observed to be particularly effective in catalyzing the formation of new peptide bonds (Fruton 1982) where amino acid esters are competing with water for the acylated papain intermediate. This will be particularly relevant to the use of papain in promoting plastein formation to restructure food proteins, as treated subsequently.

The following discussions will be restricted to brief considerations of acyl transfer reactions of lipases, papain, and pepsin in the modification of food constituents and how enzymes can be genetically engineered to control their actions.

LIPASES

Under the usual conditions of reaction, lipolytic enzymes act at the interface between insoluble lipids and the aqueous phase to effect hydrolysis of the various susceptible bonds present in lipids. In general, a large number of lipolytic enzymes act on a wide variety of lipid substrates. However, this discussion will be restricted to lipases which catalyze acyl transfer reactions involving mono-, di-, and triacylglycerols wherein fatty acids are covalently bound as esters. Ordinarily, lipases are considered to effect the transfer of acyl groups of the acylglycerols to water, leading to hydrolysis of fats, for example, and the

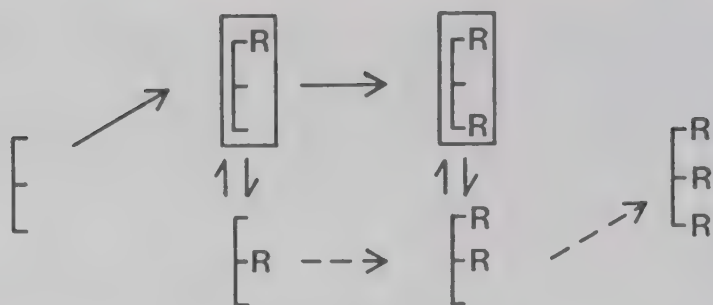
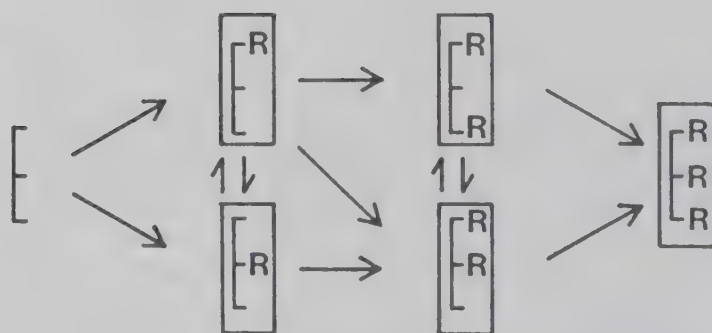
*A. niger and R. delemar**G. candidum and P. cyclopium*

FIG. 11.1. Possible reaction sequences in acylglycerol synthesis by microbial lipases. R, Acyl groups; products in boxes accumulate in reaction mixtures; solid arrows, major synthetic reactions; broken arrows, minor synthetic reactions occurring after spontaneous isomerization of 1- and 1,3-acylglycerols; reversible reactions indicate spontaneous isomerizations.

From Tsutisaka et al. (1977).

formation of fatty acids and mono- or diacylglycerols. As depicted in Fig. 11.1, the hydrolytic reaction can be substantially reversed by manipulating the conditions of the reaction. Recent research with lipases has focused on acyl transfer reactions between fatty acids and glycerol yielding a net synthesis of acylglycerols, as well as on acyl exchange reactions leading to interesterification. Most lipases seem to have a specificity for positions on the triacylglycerol molecule, although not always. For example, porcine pancreatic lipase and lipases from *Aspergillus niger* and *Rhizopus delemar* specifically cleave acyl groups from the 1,3 positions of the triacylglycerols (Brockerhoff and Jensen 1974; Okumura et al. 1976). On the other hand, the lipases from *Geotrichum candidum* and *Penicillium cyclopium* act randomly

on all three positions of the triacylglycerol molecule (Okumura *et al.* 1976). When glycerol competes with water for the acyl enzyme groups during acyl transfer reactions, the positional specificity of the lipases is also evident in the synthesis of acylglycerols (Fig. 11.1) (Jensen *et al.* 1978; Tsujisaka *et al.* 1977). These positional and stereochemical specificities of lipolytic enzymes have been exploited in the synthesis of specific lipids for research purposes (Jensen *et al.* 1978). The positional and stereochemical specificities of lipases may eventually have some technological value, particularly in catalyzing specific acyl exchange reactions.

The physical properties of triacylglycerols depend upon the chain length and unsaturation of the acyl residues in addition to the position of attachment to the glycerol of the various acyl groups (Stevenson *et al.* 1979; Tanaka *et al.* 1981). For example, the desirable thermophysical properties of cocoa butter result from palmitic and stearic acids in the 1,3 positions and oleic acid in the 2 position. Consequently, conversion of a cheaper, more unsaturated triacylglycerol to a more saturated, cocoa butter-like product might be possible as a result of specific acyl exchange reactions involving palmitic and stearic acids at the 1,3 positions, as catalyzed by appropriate lipases. Conventional technological procedures used in the edible oils industry such as hydrogenation and/or chemical interesterification are not sufficiently specific to effect the necessary changes.

Net Synthesis of Triacylglycerols

Published information on esterification reactions catalyzed by lipases is meager. Thermodynamically, lipolysis is a reversible reaction and esterification of glycerol with fatty acids should be possible. Obviously, to minimize the hydrolytic reaction, the esterifications should be carried out in the presence of as little water as possible and where glycerol and particularly fatty acids are in large molar excess.

A net synthesis of acylglycerols from glycerol and fatty acids has been catalyzed by lipases from *A. niger*, *R. delemar*, *G. candidum*, and *P. cyclopium* (Fig. 11.1) (Tsujisaka *et al.* 1977). Lipases from *A. niger* and *R. delemar* catalyzed the synthesis of acylglycerols from glycerol and fatty acids as well as from dibasic and aromatic acids. Ester bonds were formed only at the 1 and 3 positions of the glycerol in the presence of oleic acid. Using thin-layer chromatography (TLC) analyses, Tsujisaka *et al.* (1977) detected 1(3)-monoolein and 1,3-di-olein as the synthesized products from oleic acid and glycerol. However, after a long reaction time, small amounts of 1,2(2,3)-diolein and tri-olein were detected, possibly the result of spontaneous isomerization

of the former 1(3)-monoolein and 1,3-diolein to form the 2-positional isomers.

In contrast, 1(2)- and 2-monoolein, 1,2(2,3)- and 1,3-diolein, and triolein were all detected as the synthetic products from the action of lipases from *G. candidum* and *P. cyclopium*, suggesting ester bond formation at all three available positions of the glycerol molecule. Also, the synthesized acylglycerols were formed only from long-chain fatty acids. The hydrolytic course of the four lipases thus appears to be the reverse of the synthetic sequence of events, suggesting that ester synthesis proceeds via direct reversal of hydrolysis rather than by a different route. Thus, the synthesis of acylglycerols from oleic acid and glycerol is proposed to occur as depicted in Fig. 11.1. With the lipases from *A. niger* and *R. delemar*, small amounts of triolein or the 2-isomers occurred only as a result of spontaneous isomerization of the 1(3)-mono- and 1,3-diacyl enzymatic products. On the other hand, lipases from *G. candidum* and *P. cyclopium* are thought to catalyze the synthesis of ester bonds at all positions, leading to the accumulation of all possible acylglycerols in the medium.

In 16 hr at 30°C up to 75% of the oleic acid was used by the *A. niger* enzyme to form esters. Also, the *A. niger* enzyme alone was able to use 73% of added butyric acid for acylglycerol synthesis. For all the lipases, an optimum level of 80% glycerol in the reaction mixture supported acylglycerol formation to the maximum extent. On the other hand, acylglycerols were not synthesized in the presence of 95% glycerol.

In more recent studies (Linfield *et al.* 1984), lipases from *Candida rugosa* and *A. niger* utilized oleic acid, glycerol, and other alcohols to form esters. These workers drove the reaction toward esterification using a 50% excess of fatty acid and reduced water activity by distilling out water periodically at room temperature with the aid of a vacuum pump. However, if too much water were removed, the enzyme precipitated stopping catalytic activity.

In Table 11.1 are listed the yields of oleyl glycerols obtained with the lipase from *C. rugosa* after 1 hr and 6 weeks of reaction in distilled water and glycerol at 25°C. Even after 6 weeks of reaction time esterification of all hydroxyl groups was only about 80% complete. Presumably equilibrium had been obtained, although the enzymes remained highly active after 6 weeks and hydrolyzed the acylglycerol mixture overnight in the presence of water.

The enzymatic esterification of fatty acids with glycerol as currently practiced may lack practicality because the reaction is very slow. However, more active, less costly lipase preparations may become available to aid in the development of a viable process for the

TABLE 11.1. TRIOLEIN SYNTHESIS^a WITH *Candida rugosa* LIPASE AT 25°C AFTER 1 HR AND 6 WEEKS OF REACTION TIME

Component	1 hr	6 weeks
1- and 2-Monoacylglycerols	2.6 ^b	1.4
1,2- and 1,3-Diacylglycerols	4.3	19.8
Triacylglycerols	1.8	43.6
Fatty acid	91.3	35.3

From Linfield *et al.* (1984).

^a Initial molar ratio oleic acid/glycerol=5, but in heterogeneous mixture.

^b Wt % from thin-layer chromatographic analyses.

enzymatic esterification of glycerol. The major advantages of enzymatic esterifications are that they can be run at room temperature and do not involve the use of harsh chemicals or high reaction temperatures of chemical methods that may cause unwanted color formation.

Acyl Exchange

In addition to the net synthesis of acylglycerols discussed in the foregoing section, lipases can catalyze acyl exchange reactions. Acyl exchange reactions catalyzed by lipases may be of more practical value than the net synthesis reactions, since they could represent relatively simple methods for greatly altering the physical properties of existing oils by substituting small quantities of different acyl residues.

Porcine pancreatic lipase catalyzes an acyl exchange reaction between excess palmitic acid (P) and *rac*-glycerolpalmitate 2,3-dioleate (POO) to yield glycerol-2-oleate, 1,3-dipalmitate (POP), and oleic acid (O) (Stevenson *et al.* 1979). The reactions proceeded in aqueous-based systems with varying amounts of hexane added to facilitate the physical dispersion of the substrates. The formation of POP and *rac*-glycerol-1-palmitate-2-oleate (PO-OH) from POO was studied as a function of oil phase dilution with hexane, reaction time, temperature, buffer pH, and levels of P. Optimum levels of hexane dissolved added P which allowed greater incorporation of P to yield higher levels of POP and diacylglycerols enriched in PO-OH over OO-OH. Too much hexane resulted in a reduced rate of reaction, presumably because the initial reaction rate is controlled by the diffusion of POO to the reaction site. The rate of palmitate exchange was then thought to reflect not only the relative amounts of free P and O available, but also the availability of POO at the reaction site. However, under most condi-

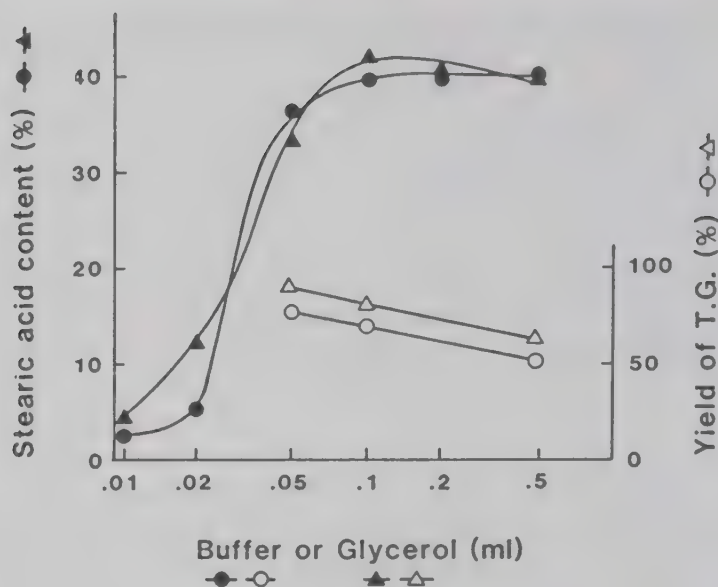


FIG. 11.2. Effect of glycerol or buffer volume on the recovery of triacylglycerols (T.G.) synthesized by lipase from *R. delemar*.

From Tanaka *et al.* (1982).

tions studied, the formation of POP appears to depend upon the concentration of P in solution in the oil-hexane phase.

In general, the acyl exchange reactions proceeded rapidly at 36°C, pH 6.0, where ~22 mol% of the POO could be converted to POP in 180 min. Presumably, the formation of POP proceeds by an initial hydrolysis of POO to PO-OH followed by resynthesis of POP in the presence of excess P.

In the foregoing study a large amount of pancreatic lipase and buffer were used relative to the triacylglycerol. In attempts to maximize the yields of triacylglycerol, Tanaka *et al.* (1981) used smaller amounts of buffer in an olive oil, stearic acid, hexane system. The lipase from *R. delemar* was used to catalyze the acyl exchange reaction. The percentage acyl exchange was defined as exchanged fatty acid in triacylglycerol/total fatty acid in triacylglycerol $\times 100$. In general, a certain minimum level of water was necessary to adequately disperse the enzyme and support the reaction. The degree of dispersion of the enzyme affected the rate of acyl exchange reaction. The addition of solids such as celite or quartz sand to the reaction mixture provided surfaces to aid in the dispersion of the enzyme and to facilitate esterification. As shown in Fig. 11.2, a reaction mixture containing celite and shaken for 3 days at 40°C yielded triacylglycerols with a stearic acid content up to about 40%. However, even in the presence of small

amounts of buffer, hydrolysis of reformed triacylglycerol occurred with a resultant decrease in yield of reformed triacylglycerol. Acyl exchange activities of 6 microbial lipases were directly related to their acylglycerol synthetic activities, again suggesting that the acyl exchange reaction consists of a hydrolysis reaction followed by reesterification of the partial acylglycerol.

In subsequent research, Yokezaki *et al.* (1982) applied immobilized lipase from *R. delemar* to interesterification of triacylglycerol in hexane. They studied the possible conversion of olive oil to a cocoa butter-like fat via interesterification whereby oleic acid moieties at the 1 and 3 positions are replaced with stearic acid. The lipase was immobilized by adsorption on celite (C-lipase) by treating the C-lipase with either a hydrophilic photo-cross-linkable resin or a hydrophobic photo-cross-linkable resin, by entrapping the enzyme in a polyurethane gel, by adsorption on porous silica beads, and by covalent binding to glutaraldehyde-activated porous silica beads. Reaction mixtures consisted of immobilized lipase (with accompanying buffer used in the preparation), olive oil, stearic acid, and water-saturated hexane. The C-lipase treated with the hydrophobic cross-linkable resin yielded 75% of the incorporation of stearic acid into triacylglycerol catalyzed by C-lipase. Free lipase was essentially inactive in the water-saturated hexane. In about 1 hr reaction time the C-lipase incorporated about 15% stearic acid into the triacylglycerols. However, under the conditions of the reaction, incorporation of stearic acid into the olive oil was linear with time up to 4 hr, at which time approximately 35% stearic acid had been incorporated into the triacylglycerols. As shown in Fig. 11.3, the immobilized lipase preparations could be used repeatedly. The C-lipase lost half of its activity after 5 days of use whereas the C-lipase entrapped in hydrophobic resin retained over 90% of its activity after 12 days of use. Although no yield data were provided, these studies indicate that immobilized lipases can catalyze the interesterification of olive oil with stearic acid in an organic solvent to produce a cocoa butter-like fat.

Reversed Micelles. In the previous studies using immobilized lipase, the enzyme is supported on a highly hydrated insoluble matrix and surrounded by an organic solvent containing the substrate. In an analogous fashion, enzymes dissolved in aqueous buffers and dispersed in a continuous organic phase to form a reversed micelle system have been studied by a number of workers (Barbaric and Luisi 1981). Thus, hydrophilic enzymes can be solubilized in hydrocarbon solvents with the help of ionic surfactants and a small amount of water. Such lipase systems may be interesting from a technological point of

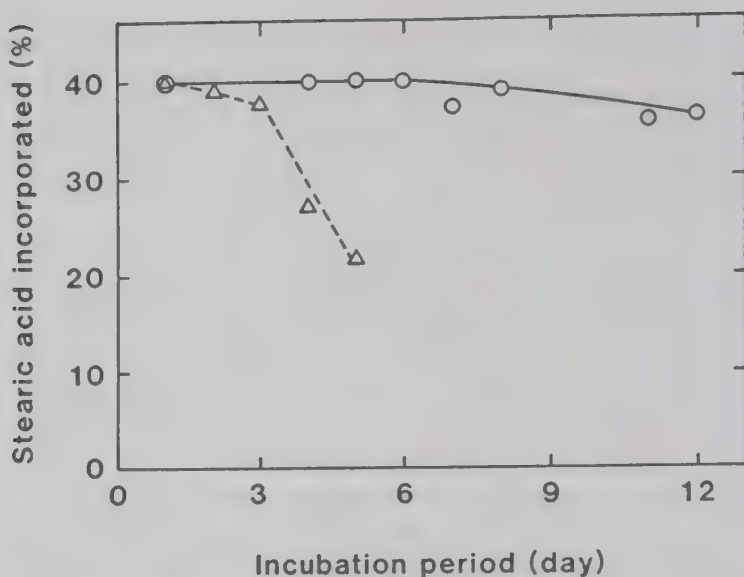


FIG. 11.3. Repeated use of lipase from *R. delemar* for interesterification of triacylglycerol. Each point represents an enzymatic reaction carried out for 24 hr. Free lipase was inactive: Δ , lipase adsorbed on celite (C-lipase); $\circ$, C-lipase entrapped in hydrophobic polymer.

From Yokozeki et al. (1982).

view in catalyzing interesterification reactions. α -Chymotrypsin, for example, has an enhanced enzymatic activity when in reversed micelles in a continuous isooctane solution which correlates with increased conformational rigidity (Barbaric and Luisi 1981). This enhanced activity, however, may be unique for α -chymotrypsin. On the other hand, a remarkable time stability observed for α -chymotrypsin in reversed micelles may be generalized to other enzymes. Thus, micellization of lipases may provide a system for modifying lipophilic substrates such as triacylglycerols.

Mechanism of Action

The manner in which lipolytic enzymes effect hydrolysis of insoluble triacylglycerols is very complex and incompletely understood. The lipase that has been studied in most detail is from porcine pancreas. There is evidence to suggest that the hydrolysis of esters by this enzyme involves an acyl enzyme intermediate. For example, acylthioester substrates yield the corresponding thiol, presumably via an acyl enzyme intermediate (Aarsman and Van den Bosch 1979). Also, by analogy with α -chymotrypsin, the action of porcine pancreatic li-

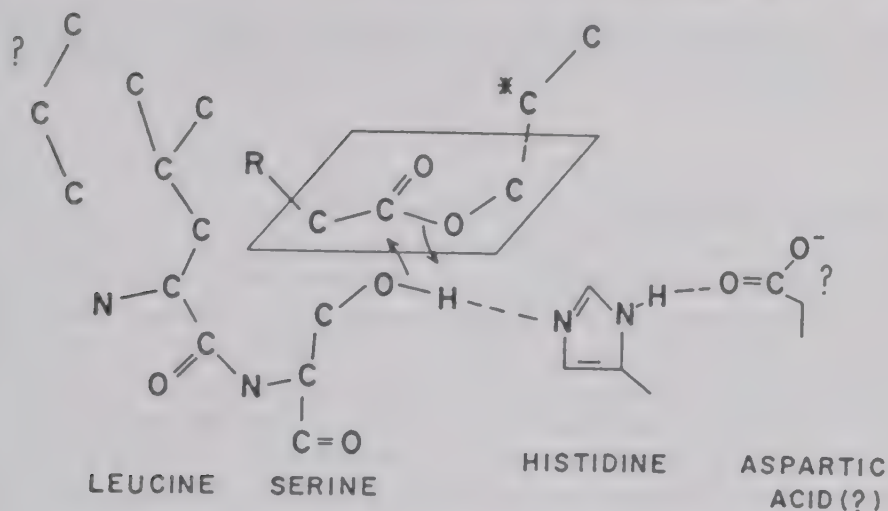


FIG. 11.4. Proposed mechanism of action for pancreatic lipase.
From Brockerhoff and Jensen (1974).

pase on *p*-nitrophenyl acetate leads to the exponential “burst” of activity thought to characterize the acylation of a reactive nucleophile in the active site (Chapus *et al.* 1976). Again, similar to α -chymotrypsin, the reactive nucleophile in the active site of porcine pancreatic lipase may be a serine residue (Brockerhoff and Jensen 1974). This burst of activity is considered to indicate that the rate-limiting step in the hydrolysis reaction is the deacylation of the acyl enzyme. In Fig. 11.4 is shown a highly speculative mechanism for the action of porcine lipase in which the nucleophilic attack on the serine oxygen of the ester bond is facilitated by a charge relay system similar to that proposed for α -chymotrypsin (Brockerhoff and Jensen 1974; Walsh 1979).

If the synthesis of ester bonds is essentially a reversal of the hydrolytic reaction, the enzyme must serve to “activate” the carboxyl group of the fatty acid, as shown in Fig. 11.4 via the formation of the intermediate acyl enzyme. Hydroxyl groups of the partial acylglycerols attack the activated carbonyl group of the acyl enzyme to yield the final acylglycerol. If the acylation of the enzyme by the fatty acid is the rate-limiting step, it might prove instructive and useful to use esters of the fatty acids in the interesterification reactions rather than the free fatty acids to more readily form the acyl enzyme intermediate. In any event, it is worthwhile to remember that the 1,3 specificity of the hydrolytic reaction of appropriate lipases is preserved in the synthetic and acyl exchange reactions, which holds forth the promise of selective modification of triacylglycerols to alter their properties. Cer-

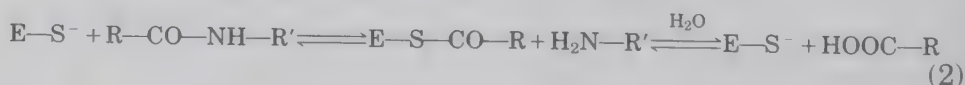
tainly, there are additional lipases with other different specificities which may prove useful for desired interesterification reactions (Jensen *et al.* 1978).

THIOL PROTEINASES

The thiol proteinases represent a diverse family of proteinases, found largely in plants, in which the thiol group of a cystein residue in the active site is the nucleophile actively engaged in acyl transfer reactions. Although there are similarities in the mode of enzymatic action among the various thiol proteinases, differences exist in details of their catalytic activities (Baines and Brockerhoff 1982; Brockerhoff *et al.* 1981, 1983A, B; Brockerhoff and Malthouse 1980). Thiol proteinases which have been studied in some detail include papaya peptidase A (Baines and Brockerhoff 1982), actinidin from kiwifruit (Brockerhoff *et al.* 1981), and, of course, ficin, bromelain, and papain (Baines and Brockerhoff 1982; Brockerhoff *et al.* 1981; Brockerhoff and Malthouse 1980; Brockerhoff *et al.* 1983A, B; Polgar 1977; Whitaker 1972).

In studies comparing the active sites of papain, actinidin, ficin, and papaya peptidase A (Brockerhoff *et al.* 1983A), it was concluded that each of the four thiol proteinases contains only one reactive thiol group per molecule. In each case the thiol forms part of an interactive system in which the nucleophilic character in the thiol group is maintained in acidic media by its association with at least one other acid-base side chain, presumably the imidazole group of histidine. There is also some question as to the participation of an aspartate β -carboxyl group in the activity of thiol proteinases (Brockerhoff *et al.* 1983A).

The details of the catalytic mechanism for papain remain to be established, particularly the way in which essential proton transfers are effected (Brockerhoff *et al.* 1981; Polgar 1977). In general, however, the reactive sulfhydryl group of Cys-25 is thought to donate its proton to His-159, thus making it a strong nucleophile in the thiolysis of amides via an intermediate thioester (Fruton 1982):



These acyl transfer reactions catalyzed by papain and their reversible inhibition as a result of thiol-disulfide interchange are crucial in development of the plastein reaction, of enzyme-modified proteins (EMP),

and of the use of thiol proteinases for tenderization of meat resulting from antemortem injection of enzyme derivatives.

Plastein Formation

It is well known that proteolytic enzymes are capable of synthesizing peptide bonds as well as hydrolyzing them (Fruton 1982). The term "plastein" has been used to signify the products obtained when a concentrated mixture of peptides (derived from proteolysis) is treated with a protease so as to effect resynthesis and/or redistribution of peptide fragments (Fox *et al.* 1982; Whitaker and Puigserver 1982). The resultant polypeptides can have substantially different physicochemical and organoleptic properties compared to the parent mixture. Since 1970 when a research group at the University of Tokyo used the plastein reaction to debitter enzymatic hydrolysates of soy protein (Fujimaki *et al.* 1970), it has become well known to food scientists as a potential method for modifying the functional and organoleptic properties of food proteins.

In the plastein reaction a proteinase is used to partially hydrolyze a protein in a relatively dilute solution. The hydrolysate is subsequently concentrated to 20–40% of peptides, and the pH of the reaction mixture is adjusted to favor restructuring of the peptides. The same protease or a different one can be used to catalyze resynthesis of polypeptides by transpeptidation or by condensation reactions. Added amino acid esters can also be covalently incorporated into the plas-teins to enrich them with nutritionally limiting amino acids and to alter the physical properties of the products (Satterlee and Chang 1982; Watanabe and Arai 1982).

Although the plastein reaction has generated considerable interest among food scientists, it may not as yet be economically attractive for applications in the food industry. Some potential applications of the plastein reaction are listed in Table 11.2. In general, the proposed changes in protein properties can result from the process outlined in Fig. 11.5. Partial hydrolysis of the protein leads to release of bound pigments and flavor components. After removal of the unwanted com-

TABLE 11.2. POTENTIAL USES OF THE PLASTEIN REACTION TO MODIFY FOOD PROTEINS

Alter solubility	Remove color, flavor, potential toxicants
Change physical properties	Incorporate amino acids to improve nutri-
Remove bitter peptides	tional and organoleptic or functional prop-
Remove unwanted amino acids	erties

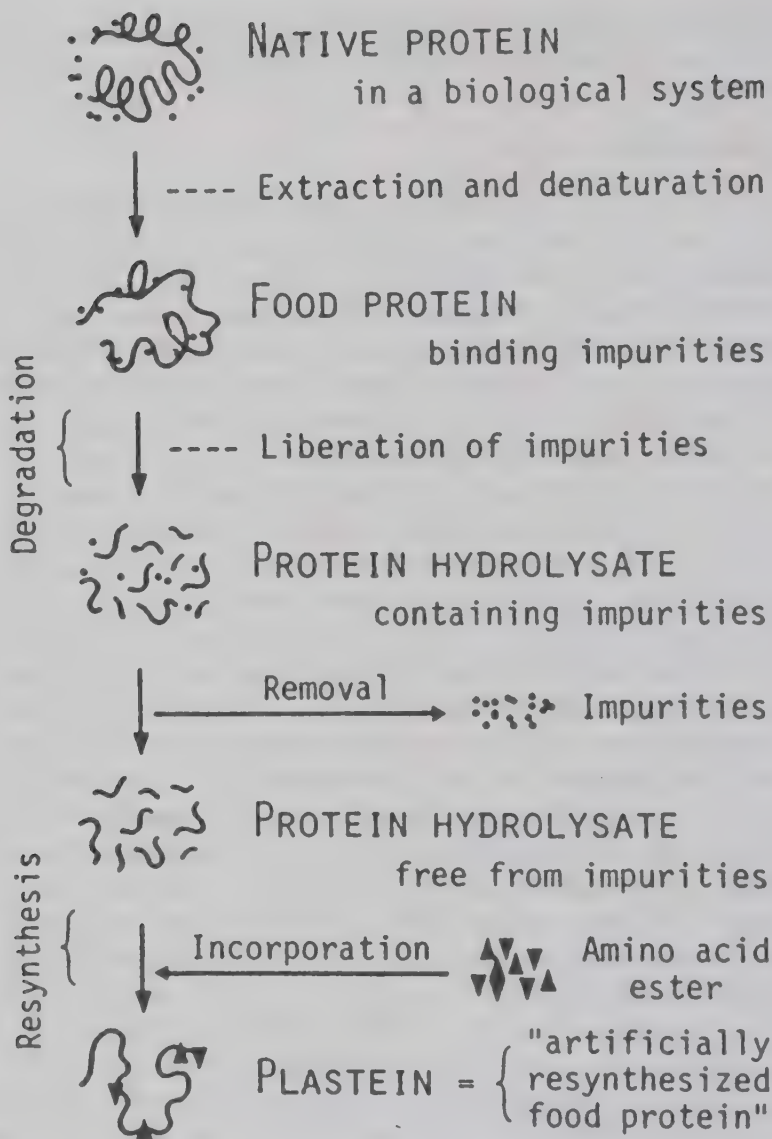


FIG. 11.5. Use of the plastein reaction to remove impurities from food proteins and to incorporate amino acid esters into the plastein.

From Fujimaki *et al.* (1977).

pounds, the concentrated hydrolysate is treated with a protease to catalyze resynthesized or rearranged polypeptide chains. Bitter peptides formed during enzymatic hydrolysis have also been removed upon subsequent resynthesis and/or recombination of peptides to yield products that are no longer bitter (Fujimaki *et al.* 1970; Whitaker and Puigserver 1982). When the plastein reaction is carried out in the

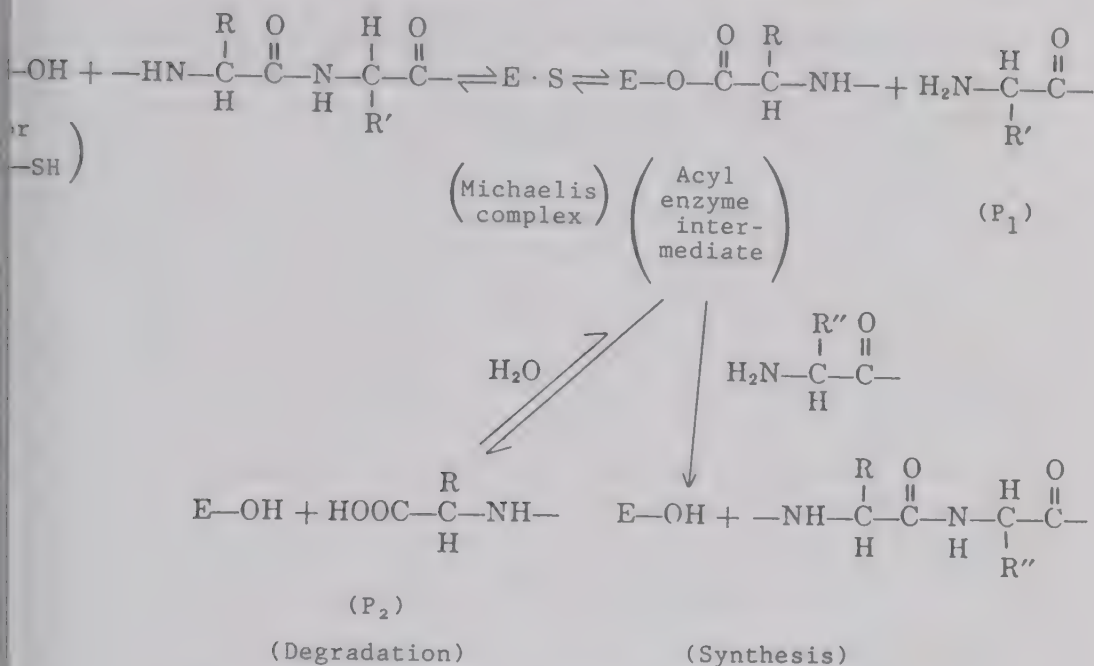


FIG. 11.6. The mechanism of the plastein reaction.

presence of appropriate amino acid esters, their incorporation can lead to improved organoleptic, nutritional, and/or functional properties of the plastein compared to the parent protein (Watanabe and Arai 1982).

The mechanism of plastein formation is depicted in Fig. 11.6. Acyl transfer reactions and acyl enzyme intermediates are key enzymatic elements in understanding the plastein reaction. During hydrolysis of proteins, the acyl group is transferred to water. However, in the resynthesis of the peptide bond the acyl group is transferred to a nucleophilic amino group of an amino acid (or its ester) or a peptide (transpeptidation). To effect a condensation reaction (reversal of hydrolysis), the enzyme would form an acyl enzyme intermediate with the free carboxyl group of a peptide, and the acyl group would then be transferred to an amino group of a participating peptide. However, it would appear that the former transpeptidation reactions would be favored over condensation reactions on energetic as well as mechanistic grounds (Fruton 1982).

There is evidence that plasteins are actually formed as a result of noncovalent hydrophobic and electrostatic interactions among rearranged shorter peptides instead of resulting from a net synthesis of longer polypeptide chains (Edwards and Shipe 1978; Hofsten and Lallasidis 1976; Monti and Jost 1979). Plasteins solubilized by oxidation

(Monti and Jost 1979) or dissolved in dissociating solvents and subjected to gel filtration (Hofsten and Lalasidis 1976) or subjected to electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (Edwards and Shipe 1978) were comprised of short low-molecular-weight peptide chains which were presumably bound together by hydrophobic bonds to form the plastein. In this case, transpeptidation reactions would result in rearrangement of peptide groups to yield short peptides whose physical properties would favor gel and precipitate formation characteristic of plasteins. Indeed, the formation of rearranged, insoluble, hydrophobic peptides could be the driving force behind plastein formation. There are indications that plasteins formed by immobilized α -chymotrypsin from peptic digests of soybean protein are more hydrophilic than those resulting from the action of soluble α -chymotrypsin (Pallavicini *et al.* 1983).

Regardless of whether plasteins are high-molecular-weight polypeptides or low-molecular-weight aggregates, they may be of some utility, particularly in improving the nutritional value of plant proteins by incorporation of lysine, methionine, and tryptophan. The physical addition of free amino acids to supplement food proteins can lead to loss of added amino acids by solubilization when the related proteins are cooked or further processed. However, covalent incorporation of amino acid esters into insoluble plasteins with acceptable organoleptic and functional properties leads to better nutrient retention (Satterlee and Chang 1982).

The classic plastein reactions are essentially a two-step process involving hydrolysis, concentration, and resynthesis. More recently, a one-step acyl transfer enzymatic process has been used to incorporate amino acid esters into proteins to yield enzymatically modified proteins (EMP) (Yamashita *et al.* 1979).

Enzymatically Modified Proteins

In the one-step process, amino acid esters are incorporated into polypeptides from proteins reacting in concentrated solutions with an appropriate enzyme. One of the most effective enzymes in catalyzing the incorporation of amino acid esters into polypeptides is papain, but at a pH of 9–10 rather than the usual neutral or slightly acidic conditions where papain is most effective in hydrolyzing proteins (Fig. 11.7) (Yamashita *et al.* 1979; Watanabe and Arai 1982). Papain is known to catalyze transamidation and transesterification reactions (Glazer 1966; Smith and Kimmel 1960) at high pH values.

In principle, amino acids could be incorporated into polypeptides in the one-step process by two routes, both involving acyl transfer reac-

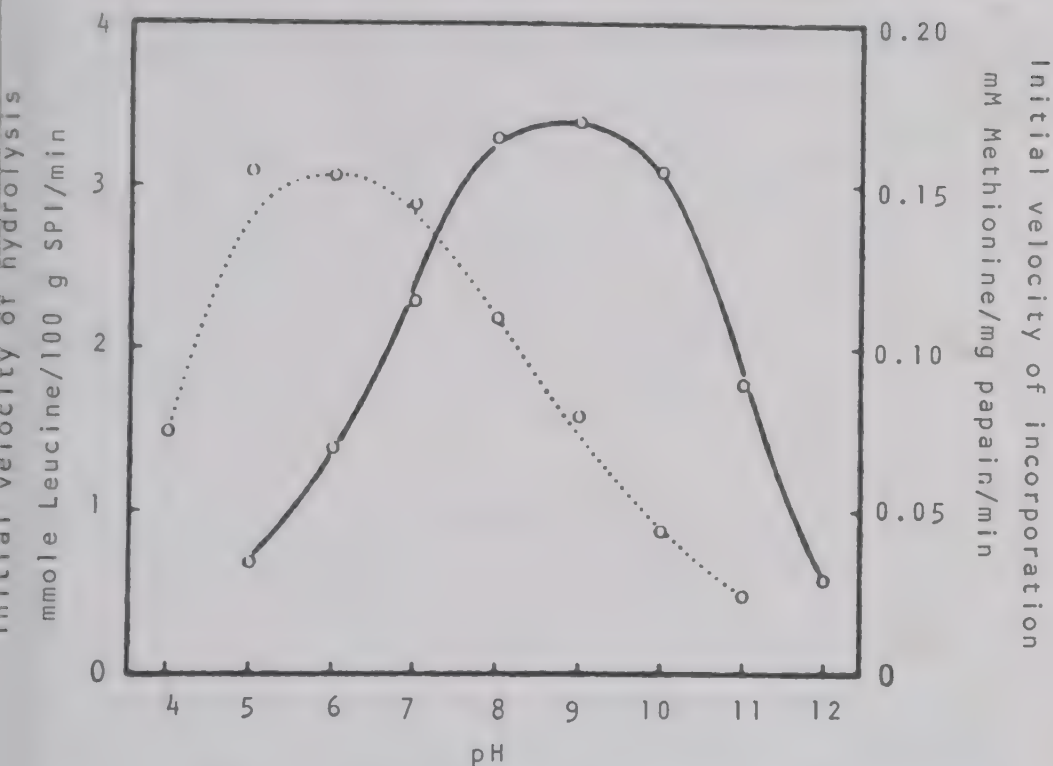
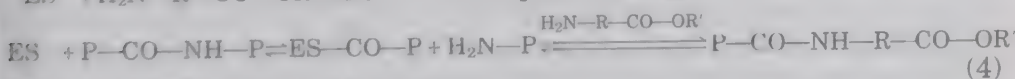
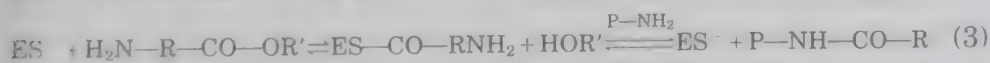


FIG. 11.7. Initial velocities of soy protein hydrolysis (dotted curve) and of L-methionine incorporation (solid curve) by papain as a function of pH.

From Yamashita *et al.* (1979).

tions (Yamashita *et al.* 1979). As shown below, the amino acid ester could react with the enzyme to yield the acyl enzyme with release of the ester group. The acyl group can then be transferred to an amino group on the protein or polypeptide, leading to incorporation of the amino acid into the polypeptide (reaction 3). Alternatively, peptide bonds in the protein can react with the enzyme yielding a peptidyl (acyl) enzyme. The peptidyl group is then transferred to the amino group of the acid ester, leading to its incorporation into the polypeptide chain. In this latter aminolysis reaction (reaction 4), the ester group remains intact in contradistinction to reaction 3. At least in part, papain catalyses reaction 4 as evidenced by the incorporation of *n*-alkyl esters of norleucine and leucine into gelatin and other proteins (Watanabe and Arai 1982).



P = protein; R, R' = amino acid moieties

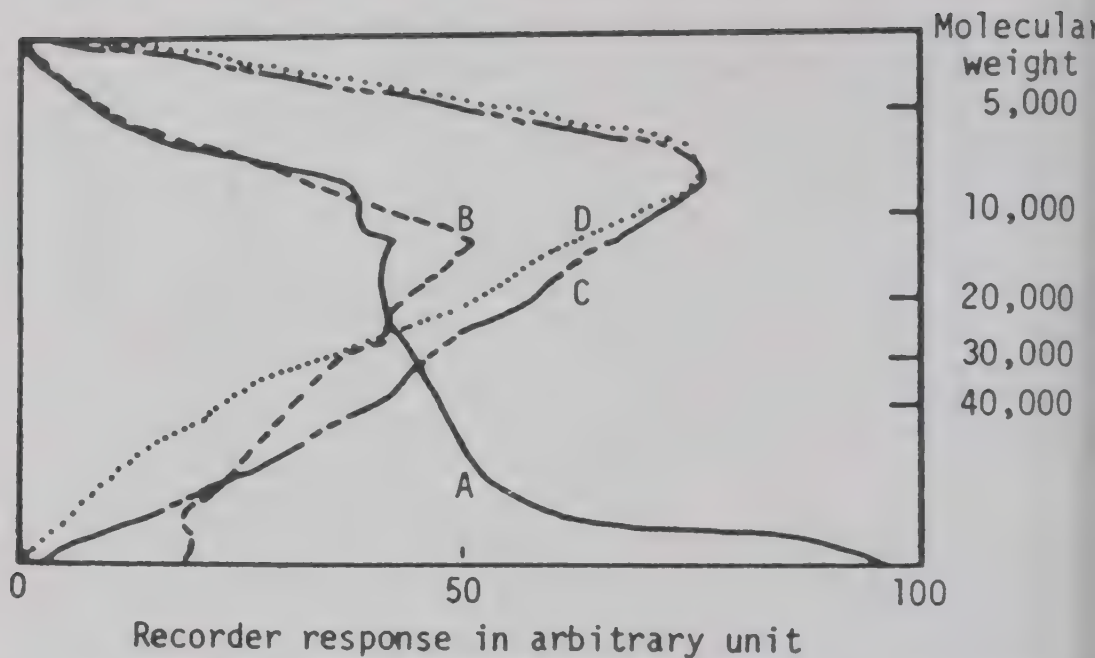


FIG. 11.8. Molecular weight distribution of the original gelatin (A), its hydrolysate (B), leucine hexyl ester-incorporated product (C), and leucine dodecyl-incorporated product (D). Molecular weight distributions determined after separating peptides in 15% of SDS-polyacrylamide gel followed by densitometric scanning of the gel.

From Watanabe *et al.* (1981C).

Moreover, as shown in Fig. 11.8, polypeptide fragments of gelatin in the molecular weight range of ~ 7500 tend to accumulate in the reaction mixture, suggesting that aminolysis with incorporation of amino acids esters occurs via peptidyl enzyme intermediate (Watanabe *et al.* 1981C).

The one-step procedure has been used to incorporate methionine ethyl ester into soy protein at pH 9.0 to enhance its nutritional quality (Fig. 11.7) (Arai *et al.* 1983; Satterlee and Chang 1982; Yamashita *et al.* 1979).

A variety of proteins have been enzymatically modified using papain to improve their functional properties, primarily those associated with enhanced surfactant activities (Watanabe and Arai 1982; Watanabe *et al.* 1982; Watanabe *et al.* 1981A, B, C). *n*-Alkyl esters of L-leucine have been incorporated in gelatin polypeptides. Approximately 1.1–1.2 mol of *n*-alkyl ester were bound to 7500 g of the product (Watanabe *et al.* 1981C). Note in Table 11.3 that those products containing C_4 – C_6 alkyl esters exhibited greater whippability whereas

TABLE 11.3. FUNCTIONAL PROPERTIES OF GELATIN MODIFIED BY PAPAIN-CATALYZED ATTACHMENTS OF L-LEUCINE *n*-ALKYL ESTERS

Alkyl chain length	Average MW	Leucine content ^a	Increment ^a	Alkyl content ^a	W ^b	F	E
Gelatin ^c	—	—	—	—	1.7	—	—
0 ^d	12,000	2.3	—	0	1.8	0.05	0.00
6	7,500	3.8	1.5	1.1	4.5	0.45	0.66
8	7,300	3.8	1.5	1.2	4.7	0.30	0.69
12	7,300	3.7	1.4	1.1	1.3	0.00	0.74

^a Mol/7500 g.^b W, whippability; F, foam stability; E, emulsifying activity.^c Unhydrolyzed gelatin.^d Control gelatin hydrolysate.

those containing C₁₀–C₁₂ esters were able to better stabilize oil/water-type emulsions. In related studies, alkyl esters of L-leucine were incorporated into succinylated fish protein concentrate, soy protein isolate, casein, and ovalbumin to yield succinylated products with high surfactant character (Watanabe *et al.* 1981A). The amphiphilicity of the hydrophilic, succinylated proteins coupled with the hydrophobic leucine esters resulted in enzymatically modified proteins with excellent whippability and emulsification properties.

Papain-Catalyzed Thiolation

Papain catalyzes transamidation and transesterification reactions in an alkaline region that is different from the usual neutral or slightly acidic conditions for protein hydrolysis (Glazer 1966; Smith and Kimmel 1960). We have seen in the previous studies how amino acid esters can be incorporated into polypeptides upon aminolysis of a peptidyl enzyme intermediate (reaction 4). It is known that papain can also catalyze the transfer of the acyl moiety of a thiol ester to an amino compound to form peptide bonds (Metrione and Johnston 1964). Sung *et al.* (1983) exploited these characteristics of papain to introduce thiol groups into soy proteins via acylation with *N*-acetylhomocysteine thiolactone (AHTL), an internal thiol ester. In this case, the enzyme is presumably acylated by AHTL, and the acyl group is transferred to amino functions of the soy protein (Fig. 11.9). As shown in Fig. 11.10, approximately 3% by weight of AHTL was incorporated into the soy protein at pH 9–10. It was not reported to what extent, if any, AHTL reacted with the protein in the absence of enzyme. Note that hydrolysis of the soy protein was very low at pH 10. The thiolation of the soy protein improved the solubility, emulsification, foam-

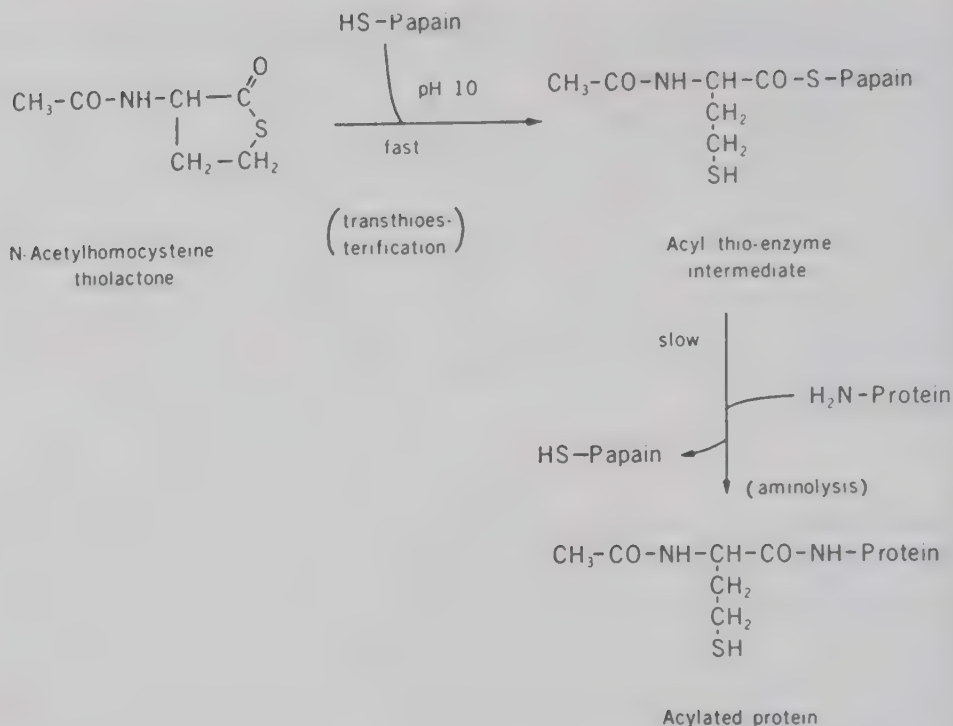


FIG. 11.9 Papain-catalyzed incorporation of *N*-acetylhomocysteine into soy proteins.

From Sung *et al.* (1983).

ing, and some rheological properties of the modified soy protein (Sung *et al.* 1983).

In general, the foregoing research indicates that acyl transfer reactions catalyzed by papain can be used to modify the organoleptic, functional, and nutritional properties of proteins. A proper understanding and manipulation of these acyl transfer reactions will allow their potential use in improving the quality of food proteins.

Reversible Blocking of Acyl Transfer

The thiol proteinases offer excellent opportunities for reversibly blocking the thiol group in the active site via thiol-disulfide interchange reactions. The reversible inhibition of acyl transfer reactions that results has been exploited in the tenderization of meat by antemortem injection of the enzyme derivative (Kang *et al.* 1974). An excellent example of this chemical latency arises out of the preslaughter

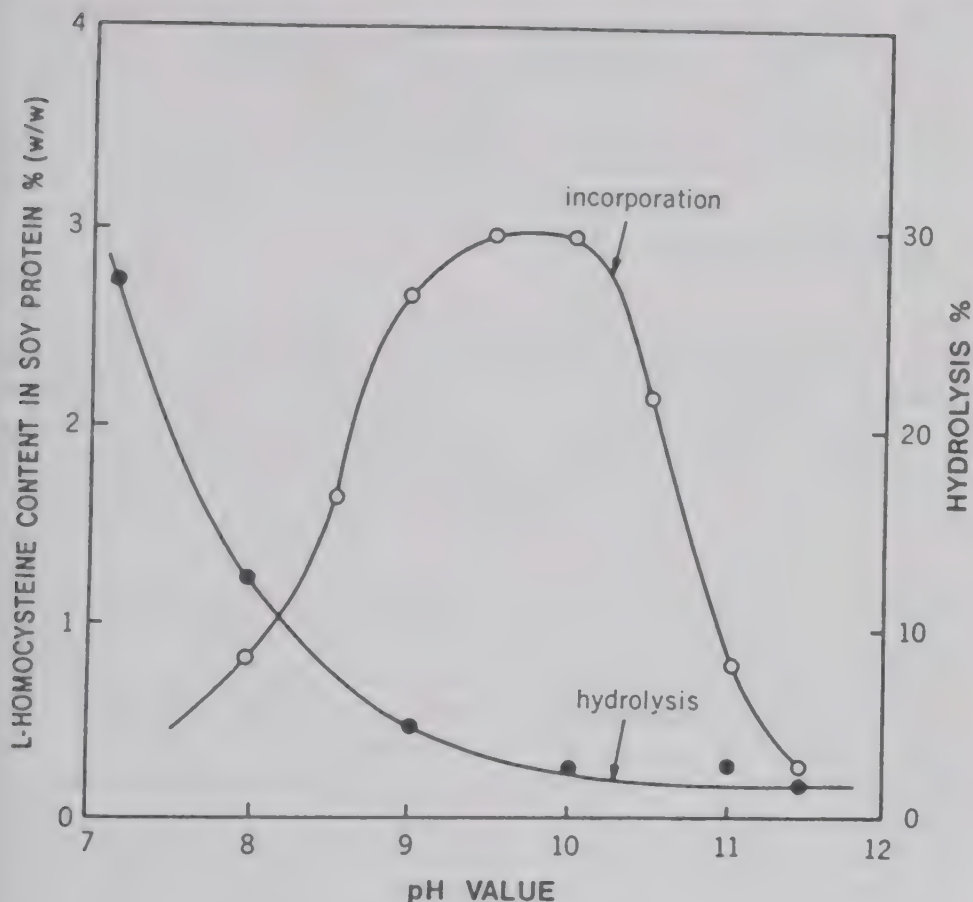
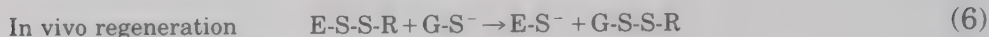
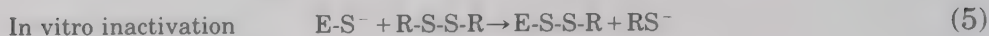


FIG. 11.10. Dependence of papain-catalyzed *N*-acetylhomocysteine incorporation into proteins on pH compared to protein hydrolysis.

From Sung et al. (1983).

intravenous injection of the sulfhydryl protease papain into animals, again to enhance tenderization of meat via postmortem proteolytic activity. In this case, the enzyme injected before slaughter of the animal is disseminated by the circulation of the animal throughout its musculature. The uniform distribution of enzyme within the tissues results in postmortem tenderization of the resultant meat. Injection of the free, active enzyme results in stress to the animal accompanied by internal hemorrhaging, leading to rejection of the carcass. However, if the essential thiol group in the active site of papain is reversibly blocked by thiol-disulfide interchange, this inactive enzyme can be administered to the animal without ill effects. The latent papain activity is subsequently regenerated in situ by reducing agents in the

meat (perhaps by glutathione while cooking) as follows:



ACID PROTEINASES

The acid proteinases comprise a large group of homologous enzymes derived from microorganisms as well as animals. These structurally related endopeptidase enzymes include chymosin, pepsin, and gastric-sin from bovine gastric mucosa, pepsin from various species including pig and chicken, and fungal proteases from *Endothia parasitica*, *Mucor miehei*, *Mucor pusillus*, *Penicillium janthinellum*, and *Rhizopus chinensis* (Bech and Foltmann 1981; Bott *et al.* 1982; Foltmann 1981; Fruton 1976; Hsu *et al.* 1977; Kay and Valler 1981). They all have acidic pH optima for proteolytic activity and, so far as is known, generally contain two aspartic acid residues as major catalytic groups of the active site (Bott *et al.* 1982). The acid proteases tend to lose activity above pH 7. In general, they cleave peptide bonds adjacent to hydrophobic amino acid residues. For example, the primary specificity of porcine pepsin on substrates of the type A-X-Y-B favors most rapid hydrolysis at the X-Y bond when X = Phe and Y = Trp, Tyr, or Phe. Significantly lower rates are observed for substrates in which X = Trp, Tyr, Leu, or Met, and Y = Leu or Met (Bozler *et al.* 1982). Although the major activity of porcine pepsin involves the hydrolysis of peptide bonds, it can under appropriate conditions catalyze condensation reactions between hydrophobic amino acids to yield hydrophobic peptides (Bozler *et al.* 1982; Fruton 1982). The primary specificity of pepsin in its hydrolytic action also applies to the condensation reactions (Bozler *et al.* 1982). Pepsin can also effect transpeptidation reactions involving apparent acyl transfer or amino transfer reactions (Fruton 1976, 1982; James *et al.* 1977).

The acid proteinases, especially chymosin, are very effective at coagulating milk and have been used extensively in the cheesemaking process. They selectively catalyze the hydrolysis of a Phe-Met bond in the κ -casein component of the casein micelle (comprised of four major caseins) to release a peptide which destabilizes these casein complexes, leading to their aggregation and the formation of cheese curd. Proteolysis by any acid proteinase retained in the cheese curd is thought to be important in subsequent changes in texture and flavor of maturing cheese. Attempts have been made to immobilize acid

proteinases for use in the continuous clotting of milk to develop a continuous cheesemaking process (Dalglish 1982). However, these efforts have been unsuccessful largely because of fouling of the immobilized enzyme beads with surface-active milk proteins and leaching of solubilized activity from the support, which has confounded results of various investigations. Perhaps a better, more definitive method of immobilization of acid proteinases might aid the development of continuous clotting processes.

Other uses of acid proteases in food-related applications are rather sparse. They may be used in some countries to chill-proof beer (Bass and Cayle 1975). Pepsin has found some use as a digestive aid. Peptic hydrolysates have been used as a preliminary step in the two-step preparation of plasteins using other enzymes to effect condensation and transpeptidation reactions (see above). An immobilized pepsin might be useful if this technology develops.

Structure-Mechanism of Acid Proteinases

The acid proteinases offer a homologous series of enzymes useful in the study of enzymatic structure and function. The primary structures, determined for a number of acid proteinases, indicate a high degree of homology in the amino acid sequences (Bech and Foltmann 1981; Foltmann 1981; Fruton 1976; Hsu *et al.* 1977; Kay and Valler 1981). Moreover, X-ray crystallographic data have revealed three-dimensional structural details for acid proteinases from *P. janthinellum* (Hsu *et al.* 1977; James *et al.* 1977, 1982) and *R. chinensis* (Bott *et al.* 1982), for example. In general, the three-dimensional structures indicate a bilobular structure with an extended hydrophobic cleft for binding of the peptide substrate (Fig. 11.11). The hydrophobic cleft can accommodate approximately eight amino acid residues, including the scissile bond. Apparently the susceptible bond is cleaved without formation of a covalent acyl or amino enzyme intermediate (James *et al.* 1977; Rich and Bernatowicz 1982).

Details of the catalytic mechanism of hydrolyses for a given acid proteinase remain to be worked out. However, it should prove instructive to consider a mechanism proposed for penicillopepsin based on its three-dimensional structure determined from X-ray crystallography (James *et al.* 1977) (Fig. 11.12).

The electron density map of penicillopepsin at 2.8 Å resolution provides the details of the active-site geometry necessary to combine with chemical and kinetic studies done with synthetic substrates to suggest a mechanism (Fig. 11.12) (James *et al.* 1977). In this particular

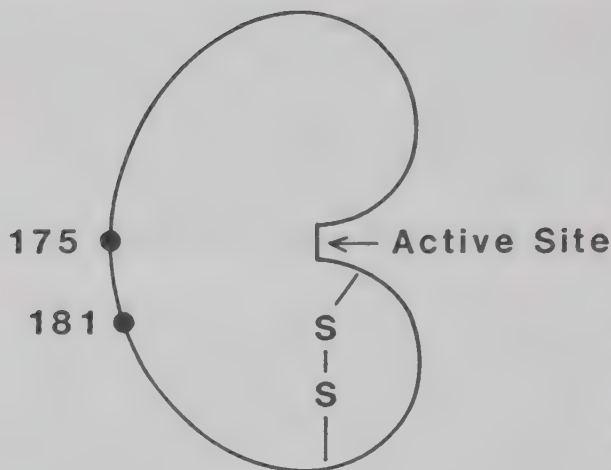


FIG. 11.11. Bilobular nature of penicillopepsin depicting peptide sequence 175–181 distal from extended active site.

From James et al. (1977).

mechanism, two β -carboxyl groups of Asp-32 and Asp-215 and the phenolic group of Tyr-75 are thought to be involved in the peptide bond cleavage. However, the β -carboxyl group of Asp-32 is too buried to act as an attacking nucleophile directly. It is also unlikely that the carboxyl group of Asp-215 acts as an attacking nucleophile, even though it is partially exposed to solvent, because the pK_a of this carboxyl group is too high at 4.7. The proposed mechanism involves general base catalysis in which Asp-32 attacks the carbon atom of the polarized $C^+ \cdots O^- \cdots H^+$ system through a bound water molecule.

As shown in Fig. 11.12, a hypothetical substrate $RCO\ NHR^+$ containing the scissile peptide bond is bound to the active site, leading to a conformational change in the enzyme, bringing Tyr-75 into proximity with the amide nitrogen of the susceptible peptide bond. Note that a water molecule lies between Asp-32 and the substrate. Polarization of the carbonyl bond is accomplished by the proton shared between the two active-site carboxyl groups. The electrophilicity of this proton is somehow thought to be associated with the nature of the substrate. The special requirement of acid proteases in having an ionized carboxylate at very low pH values results from the unusual environment of the buried Asp-32 side chain. The attack on the polarized carbonyl carbon atom occurs through the activated water molecule rather than by direct nucleophilic attack by the β -carboxylate of Asp-32. Thus, this general base mechanism of hydrolysis does not involve either an anhydride or an amino enzyme intermediate, which has been variously proposed to explain the mechanism of acid proteases. However, numerous workers have been unable to trap either of the possible covalent intermediates (acyl enzyme or amino enzyme). The pro-

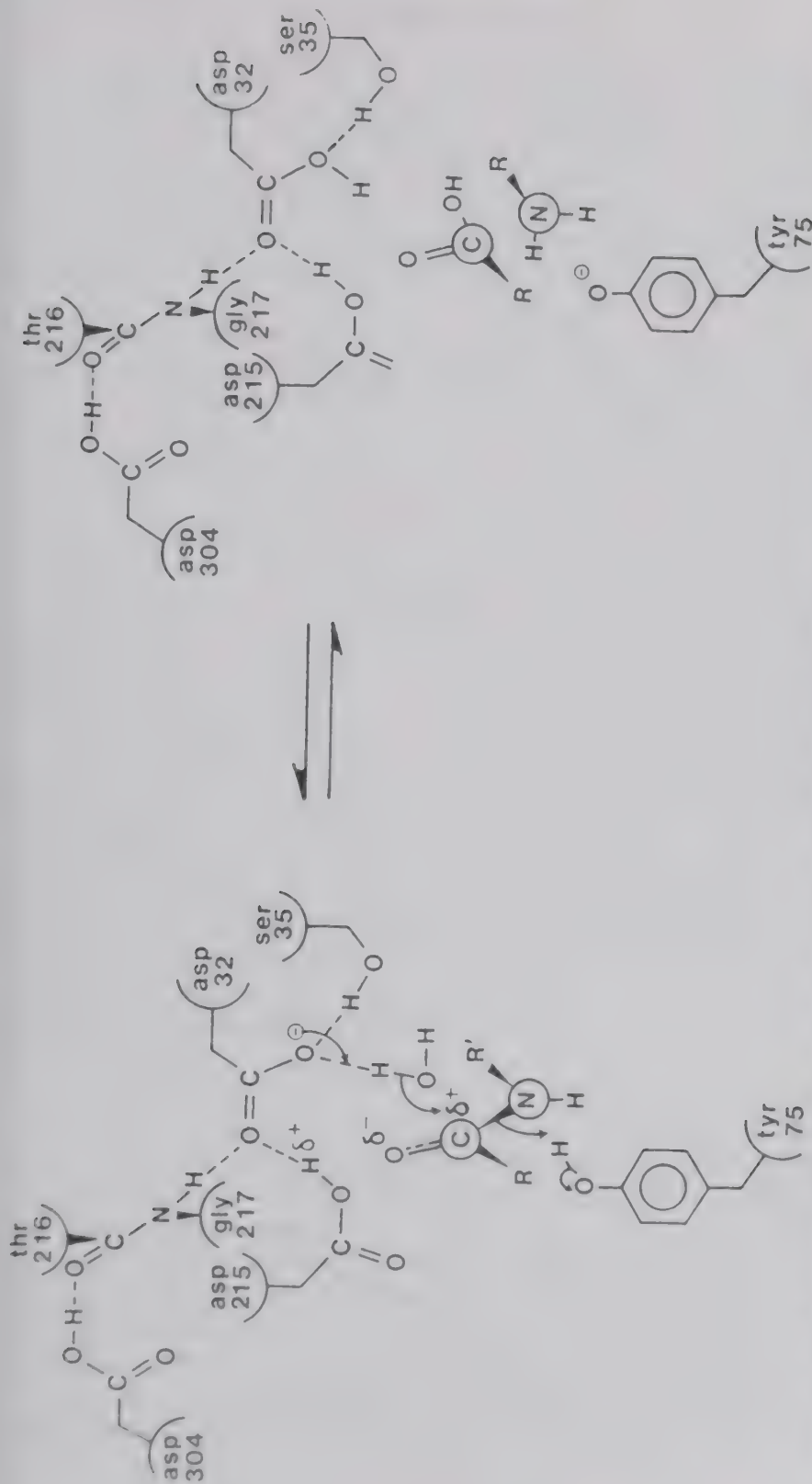


FIG. 11.12. Proposed mechanism of action for penicillopepsin illustrating absence of amino or acyl enzyme intermediates. Water is shown attacking the scissile bond after polarization of the peptide carbonyl.
 From James et al. (1977).

posed mechanism obviates the requirement for a covalent intermediate and postulates a ternary complex of enzyme, acyl fragment, and amino fragment after cleavage of the susceptible peptide bond. The order of release of products is determined by the relative affinities of the acyl or amino fragments for their respective binding sites. Fragments retained more tenaciously would tend to engage in transpeptidation reactions with incoming acyl or amino moieties of peptides, as discussed below.

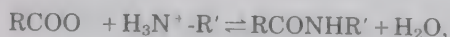
Although Tyr-75 was originally thought to protonate the amide nitrogen of the scissile bond to facilitate leaving of the amine fragment, recent research suggests that when a good substrate binds to penicillopepsin, the side chain of Tyr-75 is not directly involved in the catalysis, but enhances binding interactions with appropriate substrate side chains (James *et al.* 1982; Rich and Bernatowicz 1982).

In an analogous mechanism of action proposed for the acid protease of *R. chinensis*, a tetrahedral complex is formed by general acid–base catalysis with Asp-35 (Asp-32 in pepsin), abstracting a proton from a bound water molecule (Bott *et al.* 1982). Similar to the foregoing mechanism, the hydroxide ion undergoes nucleophilic attack at the carbonyl carbon while Asp-220 (Asp 215 in pepsin) polarizes the carbonyl bond. The carbonyl function of an adjacent glycine residue is thought to facilitate necessary proton shifts to the amide nitrogen.

It thus appears that cleavage of peptide bonds by acid proteinases does not involve intermediate covalent acyl or amino enzymes. A general acid–base catalysis results in activation of a water molecule by the aspartic acid residues in the active site. The putative hydroxide ion attacks the polarized carbonyl group of the peptide bond, thereby effecting its cleavage. Selective binding of resultant acyl or amino terminal fragments can lead to transpeptidation reactions characteristic of acid proteinases. Thus, the extended active site with binding sites for fragments on either side of the scissile bond plays a crucial role in synthetic reactions.

Acid proteinases, more especially pepsin, catalyze condensation reactions as well as transpeptidation reactions (Bozler *et al.* 1982; Fruton 1982; James *et al.* 1977). Transpeptidation usually proceeds at pH values greater than 4.0. In the general acid–base catalysis discussed above, the most likely products are the carboxylate (R-COO^-) and the protonated amino group ($\text{H}_3\text{N}^+\text{-R}'$). In Fig. 11.12, the proton on the carboxyl group would be transferred to the amino group, yielding a positively charged amino group stabilized by the phenoxide ion of Tyr-75 (or other possible negative charges in the active site). At pH values at which transpeptidation occurs (~ 4.0), the equilibrium constant

for the reaction,



is 1, and the reversal of the hydrolytic step is equally probable (Bozler 1982; Fruton 1982). However, at pH values close to the optimum pH of pepsin (pH $\sim$ 2), one would expect the carboxyl of the acyl fragment to be protonated, thus precluding transpeptidation at low pH.

The formation of short hydrophobic peptides would tend to be favored by transpeptidation reactions (Bozler 1982). James *et al.* (1977) used their mechanism to explain the distribution of transpeptidation products from pepsin acting on ^{14}C -Leu-Tyr- ^3H -Leu. Approximately 80% of the resulting Leu-Leu is ^{14}C -Leu- ^{14}C -Leu; the remainder is ^3H -Leu- ^3H -Leu with no mixed radiolabeled product. Apparently, the tripeptide binds initially so that the N-terminal Leu-Tyr bond is cleaved. Exchange of the C-terminal Tyr-Leu with a second Leu-Tyr-Leu occurs more readily than the exchange of the bound N-terminal ^{14}C -Leu product from the first hydrolysis. Through a reversal of the mechanism depicted in Fig. 11.12, Leu-Leu-Tyr-Leu is formed and subsequently cleaved by a second hydrolytic step to give ^{14}C -Leu- ^{14}C -Leu.

Residual acid proteases in cheese stemming from the coagulant can yield peptides with a bitter flavor. The bitter peptides are thought to arise from hydrolytic cleavage of the caseins, particularly the C-terminal portions of β -casein (Visser *et al.* 1983, and references cited therein). Although there is virtually no evidence that transpeptidation reactions occur in cheese to generate bitter peptides, this possibility could be studied easily with addition of radiolabeled amino acids and peptides to cheese products followed by isolation of any new radiolabeled peptides. Precipitation of or binding of hydrophobic, bitter peptides to the caseins could serve to favor their formation.

Genetic Engineering of an Acid Proteinase

We are now entering the era when it has become possible to genetically engineer enzymes (Maugh 1984). With appropriate techniques one can now systematically alter the primary amino acid sequence of an enzyme which could lead to changes in the active site, the K_m , the V_{max} , the isoionic point, the thermal stability, the three-dimensional structure, and so forth of an enzyme. Advances in molecular biology have thus opened the way to tailor enzymes to meet the requirements of the food scientist.

This brief discussion will outline a method for systematically changing the primary structure of proteins in general and enzymes

TABLE 11.4. SIX STEPS TO RESTRUCTURE ENZYME PROTEINS

-
1. Clone relevant gene in appropriate vector
 2. Expression of cloned gene
 3. Determine DNA sequence of gene
 4. Chemical synthesis of an oligodeoxynucleotide
 5. Oligodeoxynucleotide-directed in vitro mutagenesis
 6. Identification of mutant colonies
-

From Dalbadie-McFarland et al. (1982).

in particular. It will include a simple illustration of how the technique might be used to develop an oriented immobilized acid proteinase for continuous hydrolysis of proteins or continuous coagulation of milk.

A short introduction into the principal strategy used to genetically engineer enzymes will prove helpful. In Table 11.4 are listed the six major steps currently required to alter the primary structure of a protein (Dalbadie-McFarland *et al.* 1982). Limitations of space prevent a detailed discussion of the first four steps in the sequence, and the following remarks will largely be restricted to steps five and six which involve the actual events for protein modifications. Of course, one should know as much as possible about the protein in question, including its three-dimensional structure.

Principles for cloning of relevant genes into a desirable vector and the expression of the cloned gene to produce proteins in an appropriate organism have been reviewed extensively (Old and Primrose 1980; Watson *et al.* 1983). Suffice it to indicate that the sequence of foreign DNA spliced into the vector acting as a vehicle codes for the primary sequence of the protein as mediated by the intervening complementary messenger ribonucleic acid (mRNA) and transfer ribonucleic acid (tRNA). Thus, the latter mRNA and tRNA act upon the instruction of the parent DNA which dictates the primary sequence of the resultant protein. If the nucleotide bases in the parent DNA are changed (mutated), the alterations are reflected in a change in the primary sequence of the protein. Specific DNA sequences, additional to the parent gene, that control expression or biosynthesis of the foreign protein must also be inserted into the vector (plasmid) adjacent to the gene to ensure eventual production of protein by transformed host cells. Once this expression vector is inserted into competent microbial cells such as *Escherichia coli*, *Bacillus subtilis*, or *Saccharomyces cerevisiae*, the stage is set for the cell to start producing the foreign protein (step 2). The DNA sequences cloned into various vectors can be removed or replaced at will with the aid of commercially available enzymes. It is now possible to accurately se-

quence the bases in replicated cloned DNA removed from host cells using the method of Sanger *et al.* (1977) or Maxam and Gilbert (1977) (step 3). The sequence of the DNA can be used to verify that it indeed codes for the primary sequence of the protein in question. Moreover, once the sequence of the DNA insert is known, it can then be systematically altered to change the primary sequence of the protein. This brings us to site-directed oligonucleotide mutagenesis. As used by Dalbadie-McFarland *et al.* (1982), this technique relies on the synthesis of an oligonucleotide fragment of the coding strand containing one or two base substitutions, resulting in desired mutations. As shown in Fig. 11.13, a plasmid vector is nicked with a restriction enzyme. Exonuclease III is used to digest away a portion of the coding strand at the 3' end. A synthetic oligonucleotide 16–19 nucleotides in length is sufficiently complementary to the template so as to hybridize with the appropriate sequence. A number of techniques are available for oligodeoxynucleotide synthesis (Watson *et al.* 1983). Because DNA polymerase enzymes require a double strand segment for initiation of DNA replication, the synthetic oligodeoxynucleotide primes DNA synthesis and is itself incorporated into the resulting heteroduplex molecule. After molecular cloning, semiconservative *in vivo* replication of this heteroduplex gives rise to homoduplexes whose sequences are either that of the original wild-type DNA or that of the synthetic oligodeoxynucleotide. Colonies are then screened with the ^{32}P -labeled synthetic oligodeoxynucleotide primer as a hybridization probe which allows easy detection of the desired mutant. With this method it is possible to identify mutants containing single-base mismatches; however, differences of two bases between mutant and wild type (as shown in Fig. 11.13) greatly increase the ability to identify mutants unambiguously.

Dalbadie-McFarland *et al.* (1982) used oligodexy-directed mutagenesis to make a specific change in the β -lactamase gene of pBR322 in *E. coli*. In this case, the Ser–Thr diad in the active site of the enzyme was inverted to Thr–Ser to generate a mutant sensitive to ampicillin, since it did not elaborate active β -lactamase to hydrolyze the ampicillin. All of the mutants that showed strong hybridization with the ^{32}P -labeled synthetic probe were also sensitive to ampicillin and presumably contained inactive β -lactamase as a result of the mutations. The inversion of Ser–Thr to Thr–Ser was expected to affect catalytic activity because the substitution of the primary alcohol of Ser-70 by the secondary alcohol of Thr might sterically hinder nucleophilic attack by the hydroxyl group on the β -lactam ring of ampicillin. Alternatively, the amino acid changes also might cause conformational rearrangement in the active site, leading to inactivation.

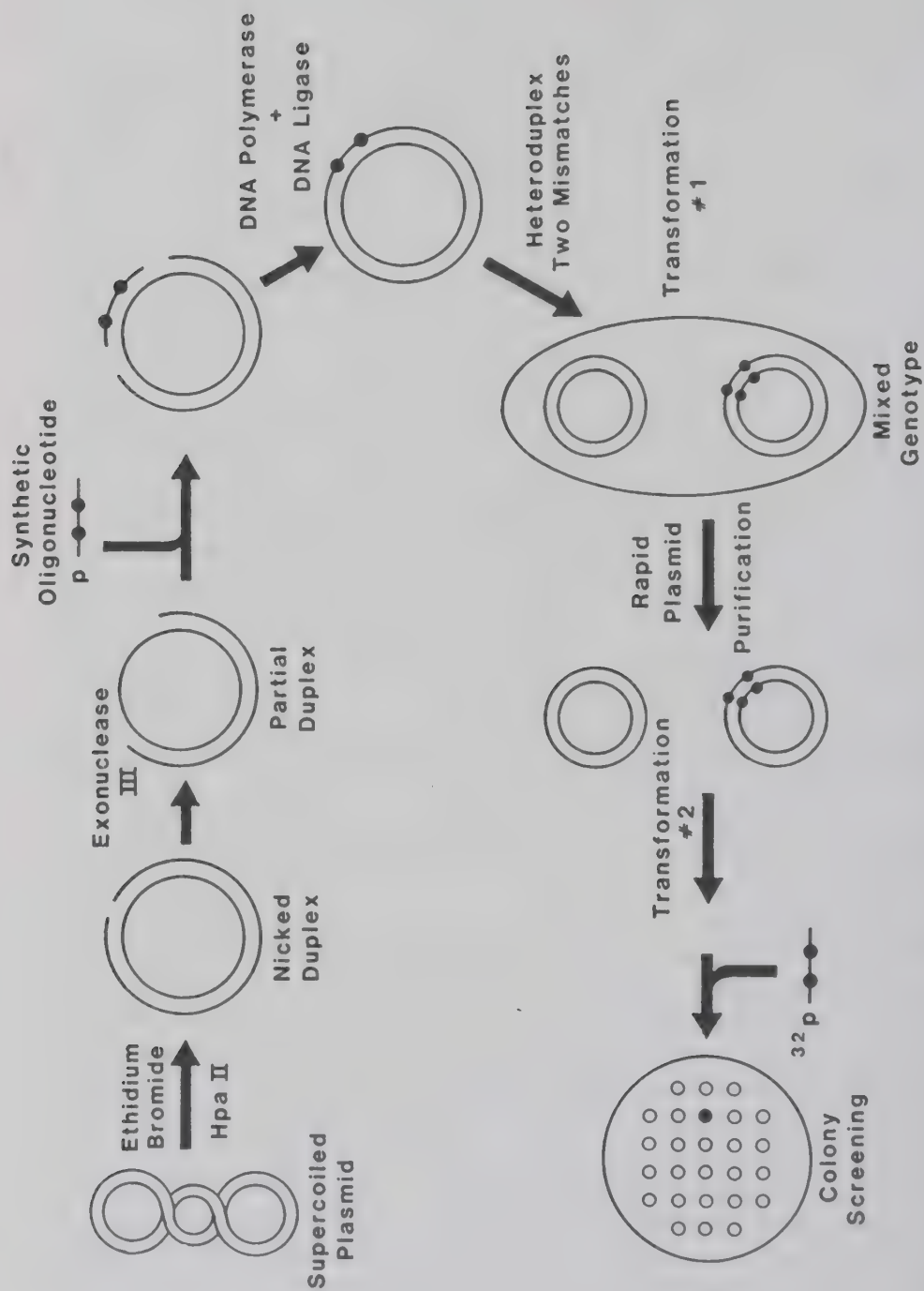


FIG. 11.13. Scheme for oligonucleotide-directed mutagenesis of double-stranded circular plasmid DNA.
From Dalbadie-McFarland et al. (1982).

SYNTHETIC

DNA PRIMER	5'	TAT	ACT	GGT	TCT	CTT	ACT	TAT	3'
					<u>TGT</u>	<u>CAT</u>			
TEMPLATE	3'	ATA	TGA	CCA	AGA	GAA	TGA	ATA	5'
DNA					<u>ACA</u>	<u>GTA</u>			
MRNA	5'	UAU	ACU	GGU	UCU	CUU	ACU	UAU	3'
					<u>UGU</u>	<u>CAU</u>			
175-181									
SEQUENCE		TYR	THR	GLY	SER	LEU	THR	TYR	
					<u>CYS</u>	<u>HIS</u>			

FIG. 11.14. Relationship of synthetic DNA primer to mRNA and amino acid sequence 175-181 of penicillopepsin.

Oligodeoxynucleotide-directed mutagenesis opens up numerous possibilities for the genetic engineering of enzymes in general. For example, a very simple single substitution of a Cys residue for a Ser residue distal to the active site in an acid proteinase could lead to a more effective means of immobilizing the enzyme so as not to obstruct the extended region binding sites of the enzyme.

Once the first three steps required for restructuring a protein (Table 11.4) have been satisfied, an oligodeoxynucleotide can be synthesized as a primer to effect the desired mutations. For example, from Fig. 11.11 it is evident that residues 175-181 in the primary sequence of penicillopepsin are distal from the extended active site. The relationship of the primary amino acid sequence in this region to a hypothetical mRNA sequence and the synthetic DNA primer is shown in Fig. 11.14. Note that Ser-178 can be changed to Cys by altering only one deoxynucleotide base in the synthetic primer. Moreover, substitution of a second base in the adjacent Leu residue can change this to a His. This latter change might act to lower the pK_a of the adjacent Cys-sulphydryl to facilitate subsequent immobilization of the penicillopepsin via thiol-disulfide interchange.

The simple substitution of a Cys for Ser-178 would not be expected to alter greatly the three-dimensional structure, hence the catalytic activity, of the enzyme. The enzyme should be immobilized readily on supports, even at acidic pH values where acid proteases would be most

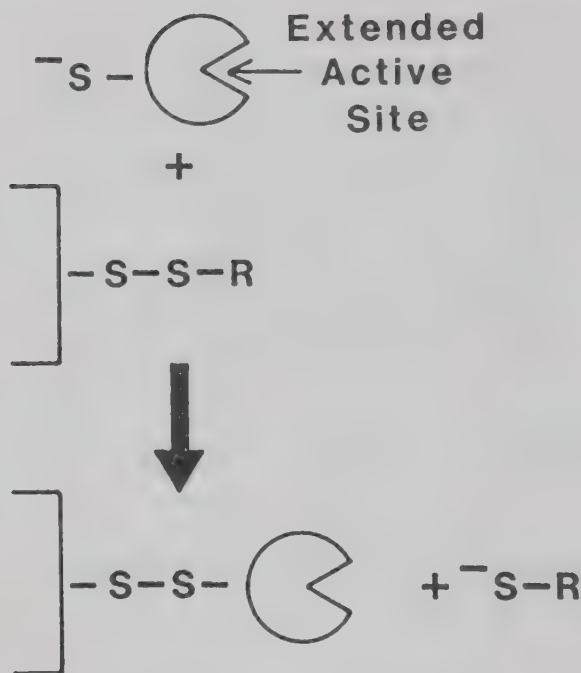


FIG. 11.15. Immobilization of engineered penicillopepsin via thiol-disulfide interchange.

stable, using the well-characterized thiol-disulfide interchange reaction as depicted in Fig. 11.15. When the thiolated support is activated with 2,2'-dipyridyl disulfide, subsequent coupling of a substituted thiol can be accomplished even at pH 2 (Svenson *et al.* 1977). In the event that there is insufficient thiolate anion to engage in thiol-disulfide interchange reactions at acidic pH values, a His residue substituted for Leu-179 might favor ionization of the adjacent thiol group of Cys-178 as follows:



The resultant greater thiolate anion concentration may thus facilitate the immobilization of the enzyme to acidic pH values. It would remain to be established whether this substitution would adversely affect enzyme activity.

In principle, all the enzyme molecules should necessarily be oriented for maximal exposure of the extended active site. This could offer advantages with macromolecular substrates or where the en-

zyme is required to act on protein aggregates, as in the continuous coagulation of caseinate particles in milk. This simple example serves to illustrate how enzymes can be genetically engineered to tailor them to our needs.

CONCLUSIONS

1. An appreciation for the synthetic and rearrangement capabilities of hydrolases can lead to the formation of novel food components.
2. We are entering the era of genetic engineering of enzymes to possibly tailor them to fit our requirements.

ACKNOWLEDGMENT

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Chemical Reactions of Proteins

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INTRODUCTION

Proteins occupy a unique status among the constituents of biological matter by virtue of their relatively large size and complex, multifaceted, and multireactive structures. These properties give them superb characteristics for functioning in biological systems in many different ways, but these characteristics also make many proteins susceptible to environmental stresses. While these susceptibilities to stresses are important in the functions of biological systems, allowing proteins to respond to or interact with the many constituents in biological systems, they are also easily attacked by a variety of chemicals and are subject to many deteriorative reactions. Indeed, susceptibility to and interaction with the environment by proteins are what make proteins highly important constituents that fulfill a major requirement for living things, namely, response to environment.

Proteins react noncovalently with substances in their environment mainly by hydrophobic forces and ionic bonds. Covalent interactions are, however, frequently encountered in normal environmental interactions such as those found between enzymes and their substrates.

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Many reactions occurring in living systems cause irreversible changes, and these deteriorative reactions are of immense importance for the proper functioning of biological systems as well as in deteriorative reactions such as are encountered in foods (for a detailed discussion of deteriorative reactions, see Whitaker and Fujimaki 1980).

Scientific investigators and industrial processors take advantage of these chemical reactivities of the protein for a variety of purposes. The purposeful modifications are generally covered under the title of "Chemical Modification of Proteins" and have been recently discussed (Feeney *et al.* 1982; Feeney and Whitaker 1982A).

GENERAL CHEMISTRY OF REACTIONS

Because of the complexity of protein structures, the chemical reactions that proteins undergo can be classified in various ways. One of the most obvious classifications is a division of modifications into those that are intentionally done and those that occur either naturally or under conditions of storage, otherwise known as deteriorative reactions. Modifications may also be classified as follows:

1. The type of chemical reaction, e.g., acylation, esterification, or oxidation
2. The type of protein side chain modified, e.g., guanidino group of arginine or imidazole groups of histidine
3. Modifications to change overall properties, e.g., physical properties by introduction of hydrophobic or hydrophilic groups, biological properties by changing susceptibility to proteolytic enzymes
4. Modifications of active or reactive site groups
5. Modifications for analytical or preparative purposes

Other classifications are also frequently used depending upon the particular subject involved.

Factors Affecting Reactions

Most chemical reactions are primarily affected by the intrinsic reactivities of the two chemical substances interacting and the conditions of the reaction. This is, of course, true with proteins, but in addition proteins have a macromolecular structure that extensively influences the reactivities of the side chains of the protein as well as the reactivities of the reagents. In addition, because of the large differences in the intrinsic reactivities of the different side chains and their different pK_s , these phenomena also have a very large influence on the reactions. Indeed, some side chain groups that should be reac-

tive to a reagent under some particular conditions are not reactive at all unless the protein is denatured or even partially degraded.

The principal reactions of protein side chains involve (1) the nucleophilic attack of the protein side chains with electrophiles such as carbonyl groups or alkyl or aryl halides, (2) Michael-type additions to unsaturated bonds, or (3) oxidations or reductions. A composite summary of some of the main reactions of the different protein side chains is given in Table 12.1. Because of the importance of the nucleophilicity on the protein side chains, the degree of ionization or protonation of these side chains is of very great importance in the reactivities. The effect of pH on the ionization of the principal side chains is shown in Fig. 12.1. These large differences in the pK_s of the side chains are frequently used to endow specificity on a particular reaction by adjusting the pH where a competing reaction is at a minimum.

There have been many monographs and texts published on chemical modification of proteins, and these can be referred to for more detail (Means and Feeney 1971; Glazer *et al.* 1975; Hirs and Timasheff 1977, 1983; Feeney *et al.* 1982).

Although there are essentially no novel reactions of organic chemistry involving protein side chains, selected examples may be helpful in depicting typical reactions (Table 12.1). Each of the reactive side chains has its unique properties, although certain reactions are in common. For a more extensive outline than that in Table 12.1, the recent review by Feeney *et al.* (1982) should be consulted. The following is an abbreviated discussion of the reactions of side chain groups.

Lysine. The amino group of lysine deserves particular attention because of its intrinsic chemistry and because of the widespread occurrence of reactions of this group in protein chemistry. Amines actually have several properties that contribute to their extensive reactivities. Of particular importance is their ability to act as nucleophiles by possessing a lone pair of electrons on the nitrogen atom. Another important characteristic is their capacity to act as bases by accepting protons from a variety of acids, including water. One of the most encountered reactions in proteins is the reaction of the amino groups with carbonyl groups (Feeney *et al.* 1975). Schiff (1901) early discovered that the condensation of a primary amine with aldehydes and ketones gives imines. There are many studies since that time, and a commonly accepted equation is shown in Eq. 1:

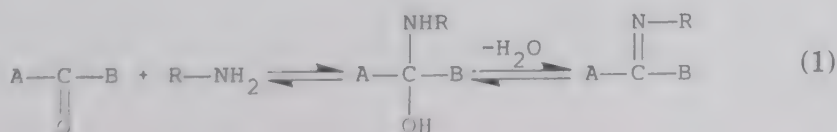
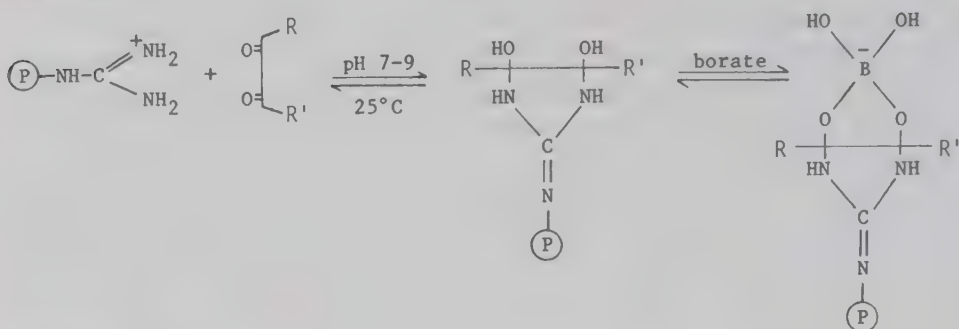
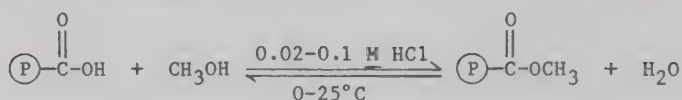


TABLE 12.1 SOME TYPICAL REACTIONS OF AMINO ACID SIDE CHAINS

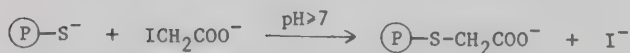
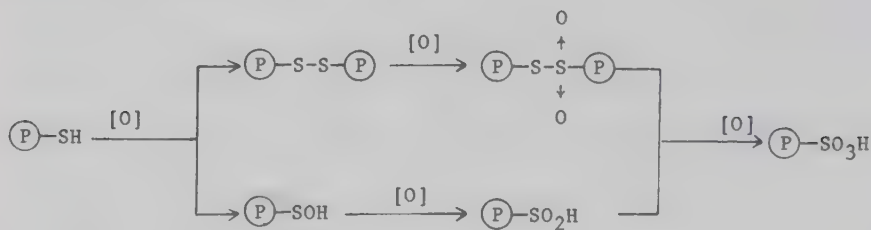
Arginine



**Aspartic
(also Glutamate)**



Cysteine



Cystine

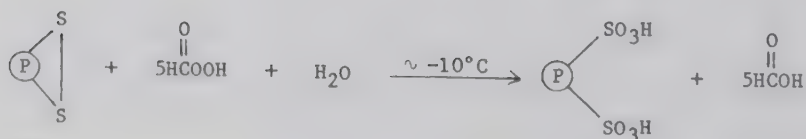
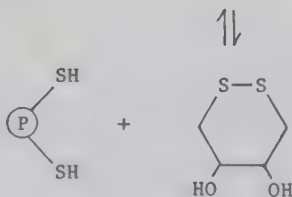
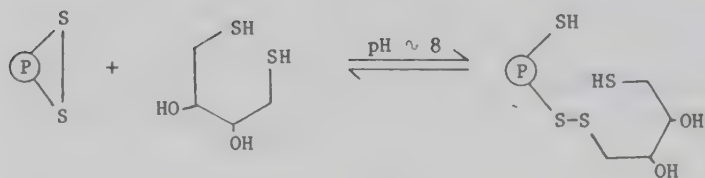
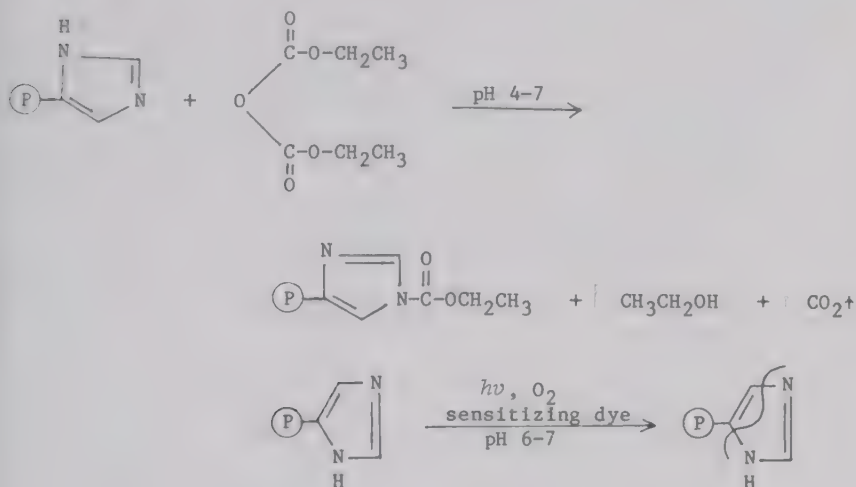
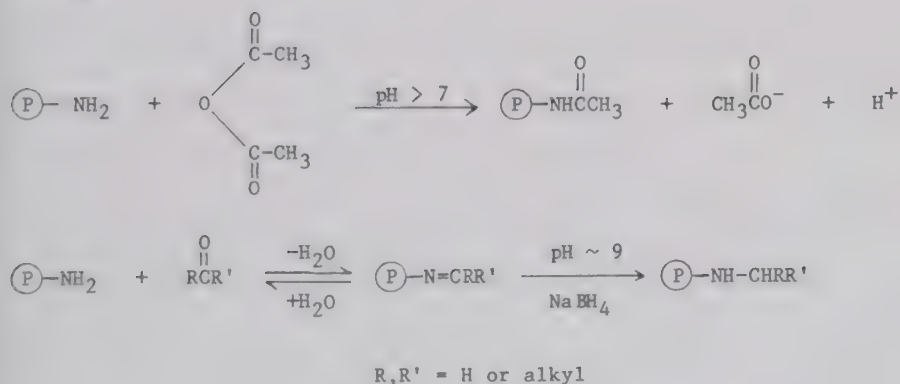
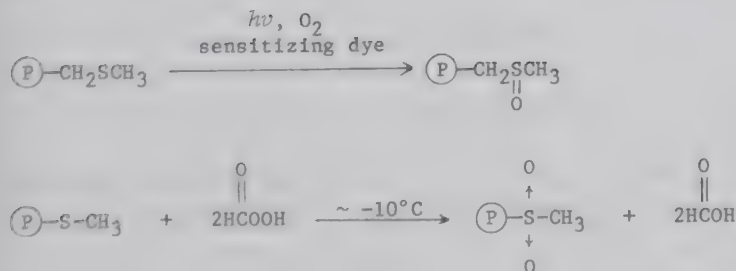
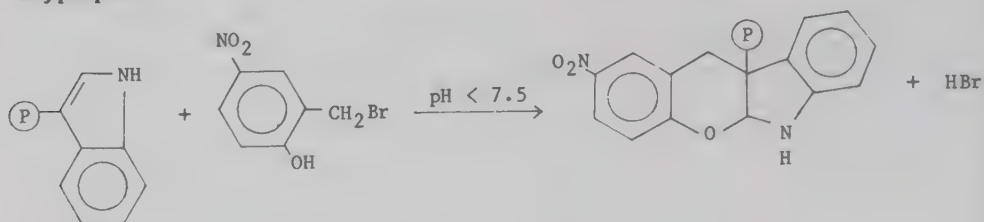
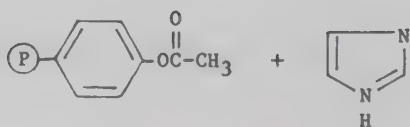
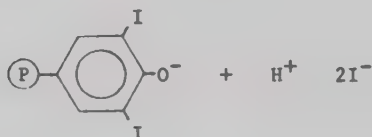
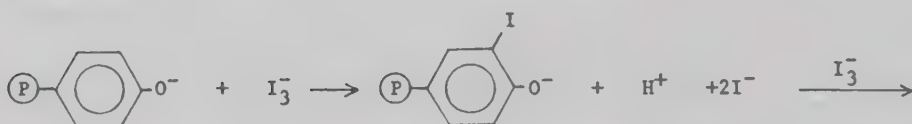


TABLE 12.1 (Cont'd.)

Histidine**Lysine****Methionine**

(continued)

TABLE 12.1 (Cont'd.)

Tryptophan**Tyrosine**

The pH of the reaction mixture has a significant influence on the rates of the carbonyl-amine reaction, and plots of rates against pH usually give a bell-shaped curve. These effects have been summarized by Jencks (1969) and further discussed by Feeney *et al.* (1975). At neutral pH a loss of water from the tetrahedral intermediate is rate determining, while at lower pH the rate of acid-catalyzed dehydration decreases, and the free amine becomes protonated. At high pH the attack and loss of free amine is fast, and the attack and loss of water or hydroxide ion can be rate determining. Schiff bases are generally considered to be the important early product of carbonyl-amine reactions. They are involved in many intermediate reactions, but the aldolamine may

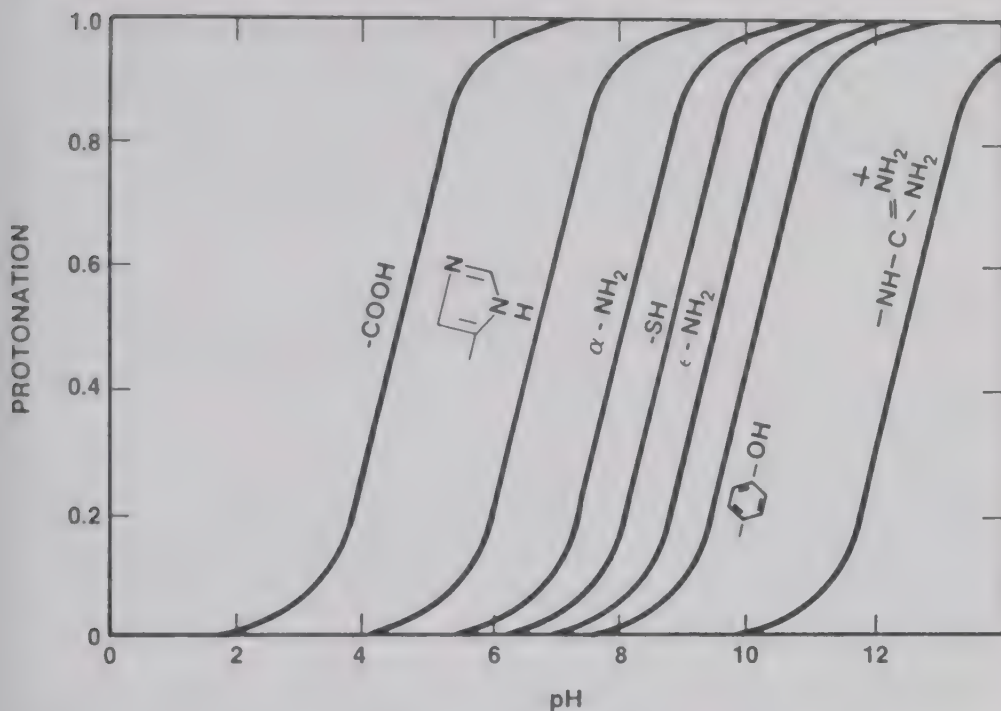


FIG. 12.1. Theoretical ionic states of amino acid side chains as a function of the pH.

From Means and Feeney (1971).

also be very important, particularly in the interaction with sugars, as is discussed below when Maillard reactions are considered.

Amino groups will also react with olefinic double bonds similar to a Michael addition reaction, giving a saturated substituted amine. In proteins, the thiol group of cysteines is usually intrinsically much more reactive (some 30 times) than the amino group, but products of amines are frequently encountered. Similarly, aryl halides or conjugated alkyl aryl halides react with amino groups as well as thiol groups by a simple bimolecular mechanism similar to the bimolecular nucleophilic displacement (S_N2).

Arginine. The guanidino group of arginine residues is especially different from other protein side chains. The group's particular structure and its strong basicity make modification difficult. Apparently the only practical and successful reagents for modifying guanidino groups of proteins are vicinol dicarbonyls. The product with some of these can be stabilized with borate through an adduct of the vicinol diols formed.

Aspartic and Glutamic Acids. The carboxyl groups of aspartic and glutamic acid have nearly identical reactivities. In the aqueous media necessary for protein studies, carboxyl groups have reactions limited mainly to the formation of esters or amides. A major reaction in protein and peptide chemistry is the formation of peptide (or amide) bonds with a water-soluble carbodiimide as an intermediate reagent.

Cysteine. Of all the protein side chain groups, the sulfhydryls of cysteine are usually the most reactive ones. They are easily oxidized to the disulfides and then to the higher oxidation states, ultimately to cysteic acid. Alkylation readily occurs and metal complexes are easily obtained.

Cystine. As the disulfide intermediate between cysteine and its more oxidized products, cystine can be both reduced to its thiol (as cysteine) and oxidized to the acids. However, other than for reduction, cystine is relatively resistant to many of the chemicals that frequently modify other protein side chains.

Histidine. In selecting the imidazole group of histidine, nature achieved a versatile reactive group for enzyme functions. With chemical reagents its nitrogens can be alkylated and its carbons can be attacked by electrophiles.

Methionine. The thioether group of methionine is easily oxidized to the sulfoxide and the sulfoxide can be easily reduced back to the thioether. Yet sulfoxides have not as yet been found to be important in protein (including enzyme) function. Alkylation readily occurs and, since the sulfur atom is unprotonated in strongly acidic solution where competing groups are protonated, alkylation can be nearly specific for methionine under these conditions.

Serine and Threonine. Acylations of alcoholic hydroxyls of serine and threonine can be easily done and are important in function in the case of the acylations of serine in some enzymes. In practice, however, these acylations of aliphatic hydroxyls are commonly not done because amino groups of lysines would be more extensively modified.

Tryptophan. Since tryptophans are usually located within the interior of native proteins and since their indole groups are intrinsically less reactive than thiol or amino groups, tryptophans are less apt to be modified by most reagents. Nevertheless, under the right conditions, they are susceptible to substitutions, oxidative cleavage, and reductions.

Tyrosine. The phenolic groups of tyrosines can either serve as nucleophiles via their ionized hydroxyl groups or can be substituted in their aromatic rings. Instability is the general rule for the hydroxyl group products, such as esters from acylation, while stability is more general for ring substitutions. With either chemical or enzymatic oxidations, cross-linkages may also occur.

Relative Reactivity of Side Chain Groups

As described earlier, the side chain groups on proteins have very large differences in reactivities, and these differences are related to the differences in intrinsic activities as well as to their pK and the structure of the protein. This applies not only to the different types of side chain groups, e.g., sulfhydryls and amino groups, but also to particular individual groups of the same type. In some cases, a particular side chain group may have a much higher reactivity than its corresponding group elsewhere in the protein or in a model system, a condition intimately related to the conformation of the protein and the properties of the adjacent amino acids.

Few chemical modifications, however, are completely specific, especially when an attempt is made to modify all of the residues of a particular group. The thiol groups of cysteines of proteins are strong nucleophiles and highly reactive to many reagents; consequently they frequently must be reversibly blocked even when attempts are made for modifying even a small number of another side chain, e.g., imidazole, guanidino, or amino.

During the past few decades, several methods have been developed for determining relative reactivity of a particular side chain group in a protein. The most popular of these employs modification with radioactive reagents and then compares the modifications of the different individual residues with time. Working models of these have been published by Kraal and Hartley (1978) in which a protein is first lightly modified by reductive methylation of amino groups (Means and Feehey 1968), with tritiated sodium borohydride as a reducing agent and formaldehyde as a methylating agent, and then compared with heavily methylated preparations using ^{14}C -labeled formaldehyde. The protein is then hydrolyzed enzymatically, peptide maps compared, and the relative amount of tritium and ^{14}C in the individual spots on the chromatograms compared. Those with high ratios of tritium to ^{14}C were peptides in which the lysines were modified the most rapidly and were therefore more reactive. Those with low ratios of tritium to ^{14}C were those modified more slowly and were therefore less reactive.

Determination of Groups Essential for Function

Determination of the essentiality of a particular group or groups in a biologically active protein is a subject of great interest to most biochemists. Chemical modification has aided considerably in this search. In most experiments, however, chemical modification reveals only which groups cannot be modified in order to prevent losses of activity rather than showing that these particular groups are essential. Two methods have been developed during the past two decades which have had high success. The first is a kinetic method (Ray and Koshland 1962), and the second is a statistical analysis (Tsou 1962). In both methods the losses in activity are compared with the numbers of groups that have been modified or unmodified during the course of a reaction. These methods can show whether one, two, or three of a particular type of side chain can be modified without loss of activity. They do not, however, identify the particular residue in the polypeptide chain. Many other methods rely on first modifying the protein in the presence of substrate, inhibitor, cofactor, etc., and then modifying it again with a radioactive reagent after the noncovalently bound substrate, inhibitor, or cofactor has been dissociated. Peptide maps may then show which groups were protected.

Still other methods rely on the affinity of a reagent to the enzyme's active center. Three main types are generally recognized (Fig. 12.2). General affinity labels are those in which the molecule contains a substrate-like structure plus a chemically reactive group; the entire

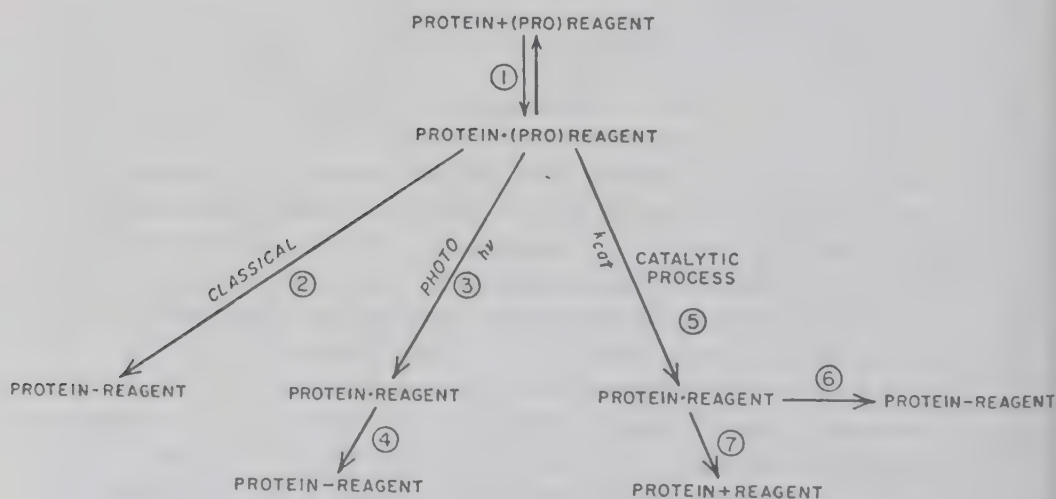


FIG. 12.2. Relationships between types of affinity-labeling reagents.
From Benisek *et al.* (1982).

molecule is then strongly attracted to the active center area by the substrate-like portion and, when in this area, the chemically reactive part modifies a group near the active site. Two further modifications of this procedure include affinity labels that do not contain a chemically active group until they are activated. One of these is the photoaffinity label. In this, the chemically active group is generated by photoactivation when the reagent is bound in the active center of the enzyme. In the other, the reactive group is enzymatically formed in the active center. This latter type of compound has been named a suicide reagent because the enzyme is considered to commit suicide by activating the reagent in the enzyme's active site. Many of these are compounds with unsaturated acetylenic groups that rearrange to conjugated double bonds which activate halides or carbonyl groups (Benisek *et al.* 1982).

SOME TYPICAL REACTIONS

There are many reactions that could be selected as typical of protein side chains, depending upon the objective, such as whether the interest is in analytical data, modification for changing physical and nutritional properties, or for active-site characterization of an enzyme. Three very different reactions with which our laboratory has had recent experience are the reductions with pyridine borane, the reactions of oxidizing agents, and the action of cupric ion on proteins. These three reactions will be discussed.

Reductions with Pyridine Borane

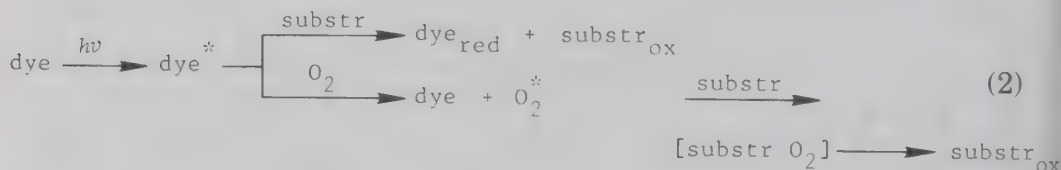
Pyridine borane complex, a general reducing agent, has been investigated recently in our laboratory as a reagent to modify proteins for two different purposes. For the one use, pyridine borane has been found a very good reagent to reductively alkylate amino groups (Cabacungan *et al.* 1982; Wong *et al.* 1984A), especially for the covalent attachment of carbohydrates. For the other use, it has been found a very good reagent for reducing tryptophan to dihydrotryptophan in strongly acidic solutions (Kurata *et al.* 1980; Wong *et al.* 1984A, B). In confirming this original report of Kurata and co-workers in which pyridine borane was shown to quantitatively reduce the tryptophans of lysozyme to dihydrotryptophan in trifluoroacetic acid, thereby inactivating the enzyme, we showed that similar treatments did not affect the activities of two proteins devoid of tryptophan, chicken ovomucoid, and bovine pancreatic ribonuclease. No other amino acids were

found to be affected by analysis of acid hydrolysates. A different usage of this acidic reduction was possible by substituting concentrated HCl for the trifluoroacetic acid. After the reduction, simply diluting the concentrated HCl to 6 *N* allowed the samples to be used directly for acid hydrolysis and amino acid analysis (Wong *et al.* 1984B). The dihydrotryptophan is completely stable to acid hydrolysis; in contrast, tryptophan is extensively destroyed (Wong *et al.* 1984B).

Several Oxidations

Three different oxidants frequently used in protein chemistry are photooxidation using a photodynamic dye, hydrogen peroxide, and sodium periodate. All three of these have been used by our laboratory.

Photooxidation was extensively developed in the laboratory of Weil (Weil *et al.* 1951). The oxidant is molecular oxygen together with visible light and a photosensitive dye such as riboflavin or Methylene Blue. The mechanism reputedly involves singlet oxygen (Eq. 2):



The most susceptible side chains are usually cysteine and histidine. Other residues such as methionine, tyrosine, and tryptophan may also be oxidized. Oxidation of the aromatics involves ring scission with a variety of products produced. Figure 12.3 shows data for photooxidation of the iron-binding protein ovotransferrin using Methylene Blue as the dye. When these data and other data on losses of activity were analyzed by the kinetic method (Rogers *et al.* 1977), it was shown that there were two histidines per binding site, either of which could not be oxidized without loss of activity.

Hydrogen peroxide is a reagent used commercially for sterilization, bleaching, and as a chemical reagent in fundamental protein chemistry. It rapidly oxidizes cysteine to the acids (Table 12.1) and methionines to the sulfoxide when the reagent is used at higher concentrations (Penner *et al.* 1983). In the presence of an organic acid, such as formic acid where performic acid is formed, tryptophans and disulfides are also rapidly oxidized (Means and Feeney 1971).

Sodium periodate has long been a reagent for the oxidation of 1,2-*cis* hydroxyls in carbohydrates, and this has received extensive usage

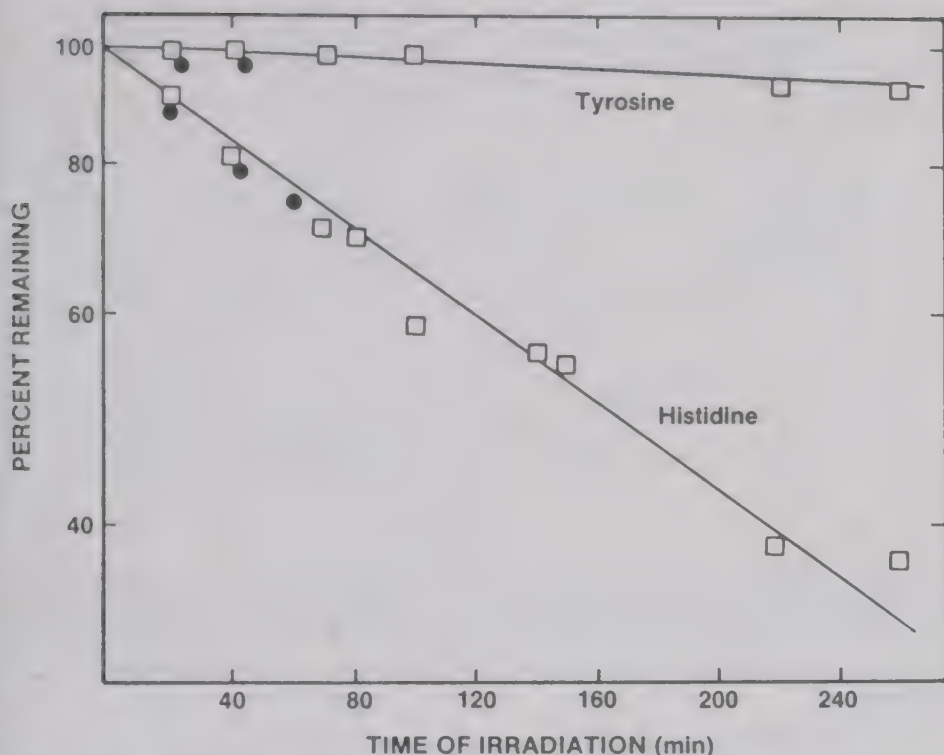


FIG. 12.3. Semilogarithmic plot of the losses in histidine and tyrosine upon photooxidation of ovotransferrin. Losses in histidine and tyrosine determined by amino acid analysis: apoovotransferrin, $\square$; diferric ovotransferrin, $\bullet$.

From Rogers *et al.* (1977).

for the oxidative removal of terminal hexoses on the carbohydrate prosthetic groups of glycoproteins. In more recent studies in our laboratories, however, periodate at substantially lower concentrations than used for removal of carbohydrates from glycoproteins has been found useful for three other purposes: the oxidation of methionines to methionine sulfoxides, the reversible removal of hydroxyalkyl substituents on amino groups, and as an affinity-like reagent for the oxidation of tyrosines in the active center of transferrins. At lower levels of periodate (5 mM), methionines were oxidized only to the sulfoxide, with no sulfones being detected (Yamasaki *et al.* 1982). When compounds such as hydroxyacetone are reductively alkylated onto lysines to give the hydroxyisopropyl derivative, these can be oxidatively removed with relatively low levels of periodate to regenerate the lysine side chain (Geoghegan *et al.* 1980). Similarly, sugars reductively alkylated via their carbonyl groups on lysines can also be removed (Wong *et al.* 1985A). In the case of the metal-binding proteins, the homolo-

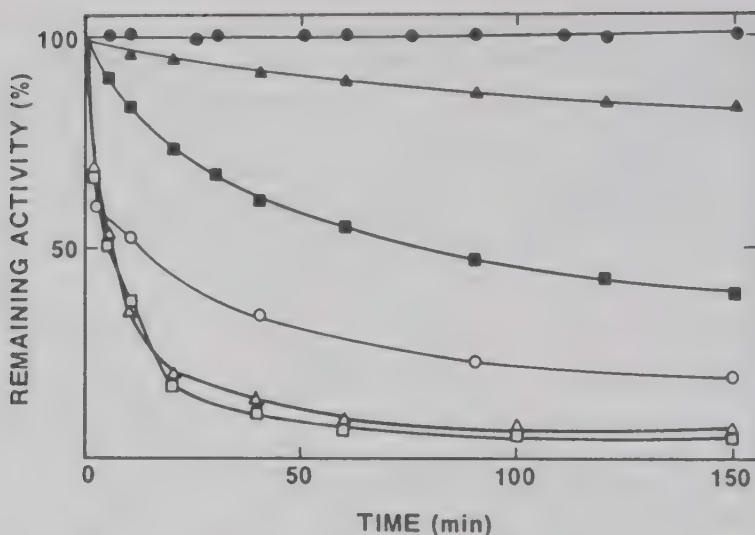


FIG. 12.4. Rate of periodate inactivation of transferrins in 0.1 M NaHPO₄, pH 8.0. Conditions were 5 mg of protein/mL and 5 mM NaIO₄: iron human lactotransferrin, ●; iron chicken ovotransferrin, ▲; iron human serum transferrin, ■; apo-human lactotransferrin, ○; apo-chicken ovotransferrin, △; and apo-human serum transferrin, □.

From Penner *et al.* (1983).

gous transferrins (chicken ovotransferrin, human lactotransferrin, or human serum transferrin), very low levels of periodate inactivate the transferrins by oxidizing tyrosines in the active center (Geoghegan *et al.* 1980; Penner *et al.* 1983; Feeney *et al.* 1983). The periodate does not inactivate the transferrin or modify the tyrosines when the oxidation is done on the iron complex in the presence of excess bicarbonate, but only in the absence of iron (Fig. 12.4). In addition, the inactivations do not occur in the presence of 5–8 M urea, i.e., under denaturing conditions, indicating that the periodate is acting as an affinity-like reagent because it requires the native form of the protein for a high affinity (Feeney *et al.* 1983).

Inactivations of Lysozyme and Chicken Ovomucoid by Cupric Ion

There are numerous examples of the inactivation of enzymes by the presence of small amounts of copper salts in the presence of oxygen. However, cupric salts have also been shown to inactivate lysozyme (Fig. 12.5) (Feeney *et al.* 1956) and several avian ovomucoids in the absence of oxygen. The reaction was apparently stoichiometric in that at least two atoms of copper per mole of lysozyme were required for extensive inactivation. A purple color accompanied these inactiva-

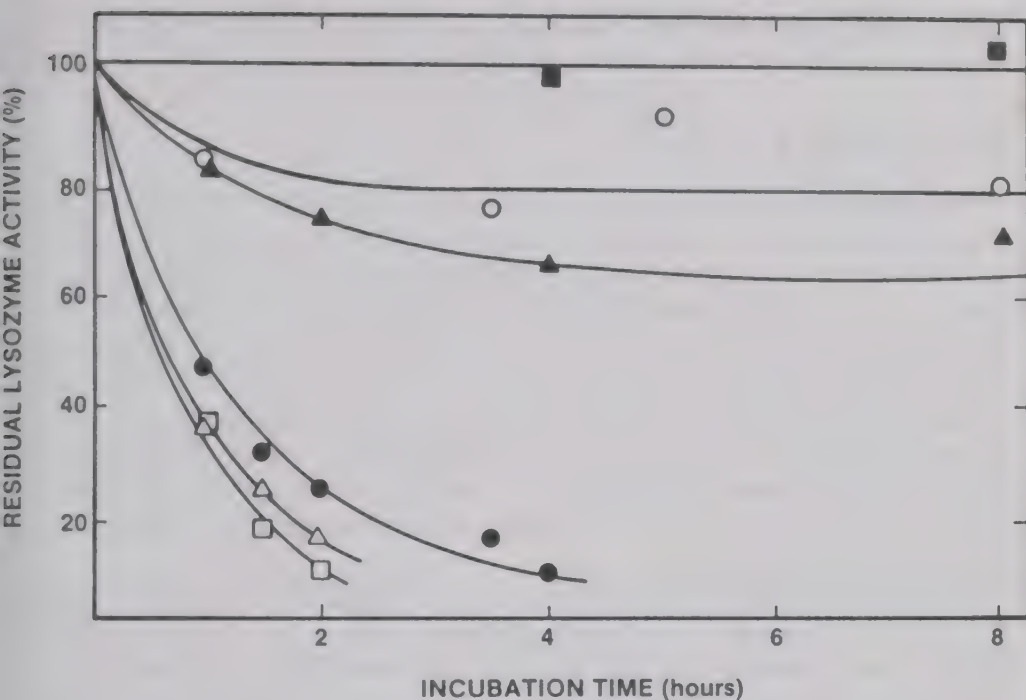


FIG. 12.5. Influence of copper concentration on the inactivation of lysozyme. The lysozyme concentration was $3.3 \times 10^{-6} M$; the temperature was $47^\circ C$; the buffer was borate adjusted to give pH 9.3: control, ■; $0.16 \times 10^{-5} M$ Cu, ○; $0.32 \times 10^{-5} M$ Cu, △; $1.6 \times 10^{-5} M$ Cu, ●; $3.2 \times 10^{-5} M$ Cu, ▲; $16 \times 10^{-5} M$ Cu, □.

From Feeney *et al.* (1956).

tions of lysozyme. The reaction went very slowly below pH 8 and was originally considered to be caused by the hydrolysis of disulfides with subsequent binding of the copper to the products, giving an apparent stoichiometric requirement (Feeney *et al.* 1956). The reaction involves both tryptophan and lysine in the more terminal phases as well as the formation of insoluble products with lysozyme, although not with ovomucoid; ovomucoid is a very soluble protein containing no tryptophan but nearly a quarter of its weight as carbohydrate. More recent electrophoretic examinations by our laboratory have shown the formation of larger molecular weight aggregates that are depolymerized by reduction, indicating disulfide interchange as part of the reaction (Wong *et al.* 1985B).

SOME DETERIORATIVE REACTIONS OF PRACTICAL IMPORTANCE

There are many deteriorative reactions involving proteins in foods, but two have received extensive interest during the past several de-

cedes. One is the Maillard reaction and the other includes those reactions caused by the exposure of the product to alkali.

The Maillard Reaction

The Maillard reaction is one of the most important and widely occurring deteriorative reactions in food. Although much is known about its chemistry, it is really so complex that only a fraction of the total reaction is clearly defined. The primary reaction is the carbonyl-amine reaction described in a previous section. In the case of the important Maillard reactions in foods, these mainly occur between the carbonyl groups of sugars and the ϵ -amino groups of the side chains of lysines in proteins. The initial reaction is followed by a series of competing and cascading reactions, including rearrangements, scissions, hydrolyses, and then even further carbonyl-amine reactions, as well as numerous other reactions.

Many articles and reviews have been written on the subject. Two extensive symposia have been published recently (Eriksson 1981; Waller and Feather 1983). A summary discussion was presented by two of the authors on the Maillard reaction and its prevention (Feeney and Whitaker 1982B).

The observed products of the Maillard reaction are the resultant of the different processes, and these are dependent on the reactive conditions. However, the critical initial reaction is still the carbonyl-amine reaction. The first product is an N-substituted glycosylamine in equilibrium with a Schiff base. Although pentoses are generally considered to be more reactive than hexoses and monosaccharides more reactive than disaccharides, these data do not indicate anything about the rates of the initial reactions and the formation of the glycosylamines. They are a result of the overall Maillard reaction and are quantitated on the basis of the type of products produced. For example, glucose exists nearly 100% in the α - and β -pyranose forms, with only a trace in the carbonyl form, and yet it is one of the more reactive sugars as judged by the degree of browning. Furthermore, fructose shares with most other ketones a relatively low rate of reaction with amines as compared to aldehydes, and yet it is considered one of the most reactive sugars in the overall Maillard reaction. The relative reactivities of glucose, fructose, and lactose for the initial carbonyl-amine reaction have been assessed by observing the coupling of these sugars to the lysine groups of proteins on reduction with cyanoborohydride (Fig. 12.6) (Lee *et al* 1979). Similar results have also been obtained with pyridine borane (Wong *et al.* 1984A).

After the initial carbonyl-amine reaction, the next step described

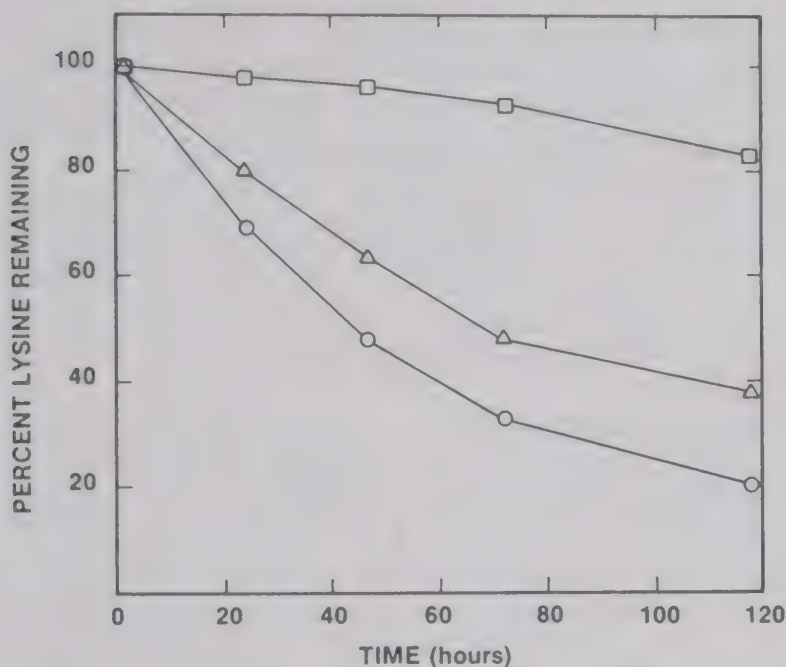


FIG. 12.6. Rate of coupling glucose (○), fructose (△), and lactose (□) to the ϵ -amino group of lysyl residues of casein in the presence of cyanoborohydride at 37°C in 0.2 M potassium phosphate buffer, pH 9.0. Losses in lysine were determined by amino acid analysis.

From Lee et al. (1979).

in the Maillard sequence is a rearrangement that may either be the Amadori or Heyns rearrangements (Feeney and Whitaker 1982B). The aldosamines are converted to a 1-amino-1-deoxyketose (in the case of glucose, to 1-amino-1-deoxyfructose) by the Amadori rearrangement, while the ketosamines are transformed to 2-amino-2-deoxyaldoses (in the case of fructose, to 2-amino-2-deoxyglucose) by the Heyns rearrangement. Once formed, these products can react with other molecules or can break down themselves. Since the Amadori and Heyns products and the many further products have been described in many of the previous papers, they will not be discussed further here.

A somewhat different interpretation of possible reactions has been advanced by Hayashi and Namiki during the past few years (Hayashi and Namiki 1980). In one of their studies they examined the reaction of glucose with *t*-butylamine and have postulated the formation of reactive two-carbon sugar fragments at a stage even prior to Amadori rearrangement and the subsequent reaction of these with the amine followed by an Amadori rearrangement to form glyoxal

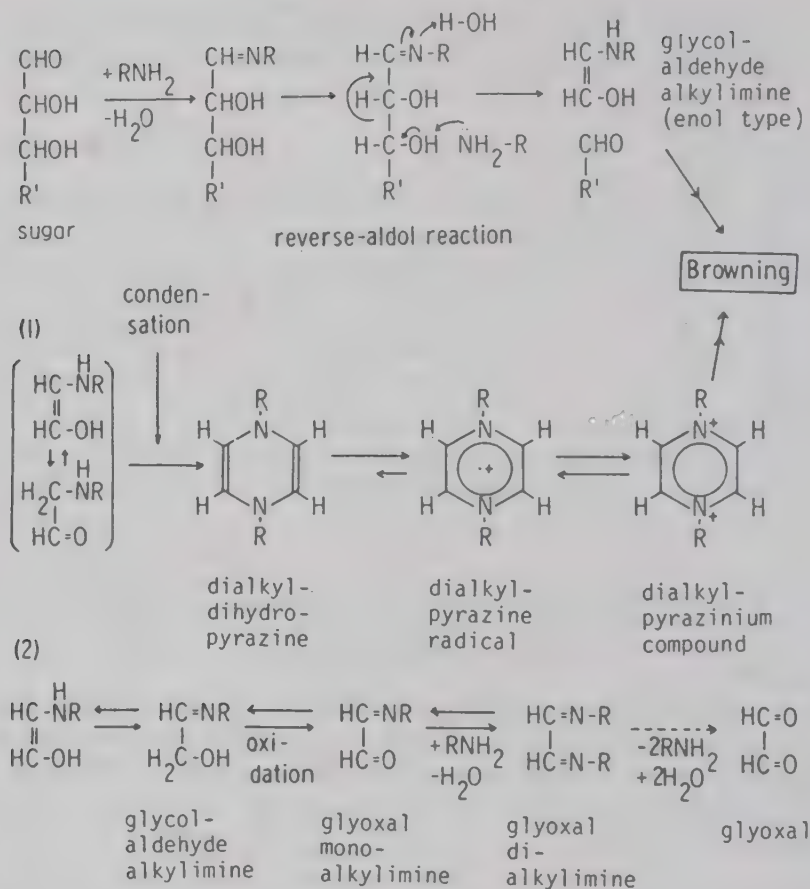


FIG. 12.7. A possible pathway for formation of the free radical product and browning in the reaction of sugar with amino compound.

Taken in part from Namiki and Hayashi (1983) and in part from Hayashi and Namiki (1981).

dialkylamine (Fig. 12.7). They have further suggested that a free radical may be formed in these reactions (Hayashi and Namiki 1981).

In general, the Maillard reaction is a deleterious deterioration that, if possible, should be avoided. Reactions in foods cause off-colors, mainly browning. In addition, the insolubilizations and loss of nutritional properties, primarily from losses in lysine, are well known in the food industry. More recently, there has been indication that there are mutagenic activities of a variety of browning products as found in the Ames test (Spingarn and Garvie 1979; Mihara and Shibamoto 1980; Shibamoto 1980; Toda *et al.* 1981). The opposite side of the argument is potential beneficial effects. Some of the colored products are desirable in some foods, as are some of the flavors produced. More recently, there has been considerable interest in the antioxidant activities.

Sometimes the changes in solubilities and physical properties of proteins are found in materials where their occurrence was not suspected. One such case has been the changes on the incubation of chicken eggs or separated egg whites for 3 or 4 days or storage at 5°C for 2 weeks. At the high pH of the egg white (approximately 9.5), the gel electrophoretic patterns of egg white proteins change significantly within 4 days at 37°C (Feeney *et al.* 1963). For this reason, individuals studying bird eggs gathered in remote areas may have encountered unexpected differences in electrophoretic patterns, patterns that might even be erroneously attributed to differences in genetic relationships.

Changes in flavor also do not need to be caused by reaction of the sugars with the amino groups of proteins. It was found over three decades ago that changes occur rapidly in the phospholipid fraction of dehydrated eggs; and these reactions between glucose and the amino groups of phospholipids were mainly responsible for the very noxious flavor and taste of dried eggs consumed by so many members of the armed forces in World War II (Kline *et al.* 1951).

Food chemists long claimed the Maillard reaction as their own, but this is no longer the case. During the past two decades, it has been found that there are other important carbonyl-amine reactions involving sugars and biological materials occurring in disease and aging (Feeney and Whitaker 1982B). Amadori products have been reported in hemoglobin (Bookchin and Gallop 1968), erythrocyte membrane protein (Miller *et al.* 1980), lens crystalline protein (Stevens *et al.* 1978), and collagen (Bailey and Robins 1973), as well as in lipoproteins of blood serum and other phospholipid-containing materials. Indeed, although the Maillard reaction is a very costly deteriorative reaction in foods, it may prove to be of even more importance to man for its deleterious effects on his own body. What drew attention to these changes in the human body was the early discovery of an abnormal hemoglobin (hemoglobin A_{1c}) (Holmquist and Schroeder 1966; Rahbar 1968; Dixon 1972) and the proof that this was due to the reaction of the blood sugar glucose with the N-terminal valine of the protein. It was H. B. F. Dixon who called attention to the fact that this was a straightforward chemical reaction (Dixon 1972). The reaction has proved to be important in monitoring diabetic patients because of the increased concentration of the derivative in these patients.

Possibly as a result of these findings related to diseases in man, during the past decade there have been many publications on the interaction of glucose and other carbonyl compounds with proteins. Amadori compounds have been reported from treatments of hemoglo-

bin with the three-carbon hydroxyaldehyde, glyceraldehyde (Acharya and Manning 1980), and the two-carbon hydroxyaldehyde, glycolaldehyde (Acharya and Manning 1981). Some of these cause cross-linking and a variety of possible products, so it is now considered that reactions with lower molecular weight carbonyl compounds may also be of great importance.

Effects of Alkali on Proteins

Alkaline conditions have long been known to cause many changes in proteins (Whitaker and Feeney 1983). Although exposure to alkali is generally a deteriorative process, it should be remembered that the hydroxide ion is a reagent in its own right. In other words, exposure to alkali can be considered as a chemical modification as well as a treatment causing deteriorations. Since exposure to alkali is discussed elsewhere in this volume and has been discussed many times by other authors and recently by two of the authors (Whitaker and Feeney 1983), the present review will only place it in the perspective of the other chemical reactions described.

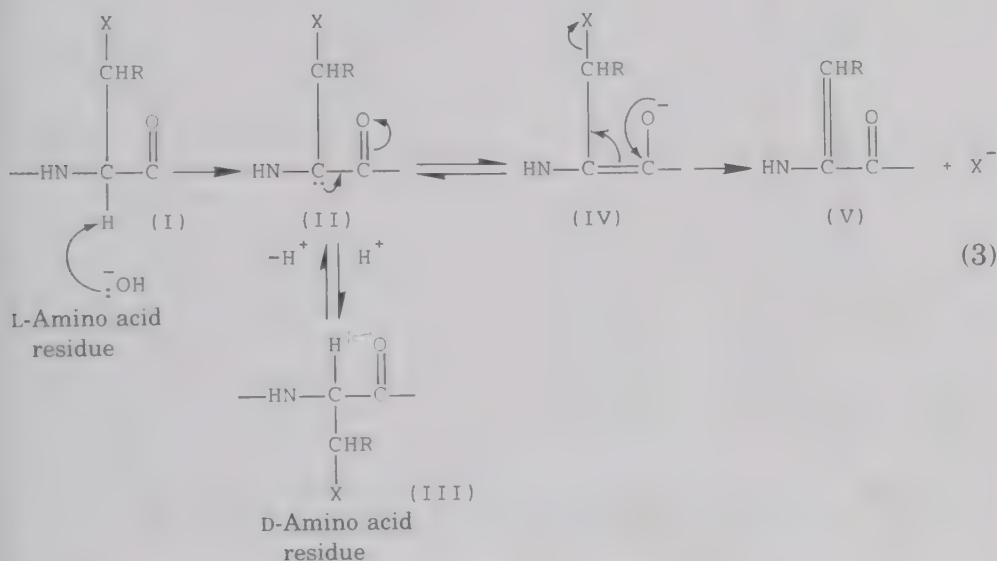
Exposure of proteins to alkaline conditions may occur during solubilization, storage of foods, the processing of foods, in the removal of toxic constituents, and even sometimes in chemical analysis and characterization. Alkali treatment causes many reactions. The more common ones are hydrolysis, elimination reactions involving the side chains of certain amino acids, racemization of amino acid residues, addition of compounds to the proteins, scission of the peptide chain, modification or elimination of nonprotein constituents (prosthetic groups, etc.), and the interaction of the protein with alkali-derived products from the environment. All of these reactions are affected by the pH, the temperature, ionic strength, presence of specific ions, and by the nature of the protein itself.

The hydrolytic effect of alkali on proteins greatly depends on the structure of the protein. More commonly, hydrolysis of amide groups (from asparaginyl and glutaminyl residues) is encountered. Hydrolysis of peptide bonds may occur readily with some proteins. With more alkaline conditions the scission of the guanidino group from arginyl residues may occur. Other reactions have been studied, particularly in model systems such as the hydrolytic splitting of the disulfide bond of cystine (Schoberl and Rambacher 1939). In strong alkali, complete hydrolysis of the protein occurs.

β -Elimination is considered by many to be one of the more important reactions of proteins in alkaline solution (Whitaker and Feeney 1983). These reactions involve the disulfides of cystinyl and the deri-

vatized seryl and threonyl (with phosphate esters or glycosidic substituents) residues. As will be discussed, lysyl residues are susceptible to this reaction because they may be subsequently derivatized by the unsaturated products produced by the β -elimination of these other groups.

A general mechanism for β -elimination is most likely that shown in the following equation:



In this equation $R = \text{H}$ or CH_3 ; $X = \text{H}$, OH , *O*-glycosyl, *O*-phosphoryl, SH , SCH_2R , or aliphatic or aromatic residues. As can be seen, the reaction can also produce the enantiomorphous amino acid by racemization. Thus, after the initial step which is the abstraction of the α hydrogen by the hydroxide ion, the carbanion formed then can either add a proton to give the enantiomorph, or the X group can be expelled to form an unsaturated derivative (V). The nature of the X group dictates what the pathway will be. For example, when X is an *O*-glycosyl, *O*-phosphoryl, or disulfide, β -elimination occurs. The unsaturated product from the β -elimination can generally be monitored by an increase in the absorbancy at 241 nm (Bohak 1964). This is generally a satisfactory method only at the very beginning of the reaction because subsequent addition reactions soon occur which remove this absorbancy. Threonyl and seryl residues are lost by β -elimination at only about 5% the rate of the loss of cystinyl residues. In the authors' laboratories these relative rates were examined by using three different proteins: lysozyme containing disulfides but no *O*-phosphoryl or *O*-glycosyl; phosvitin containing *O*-phosphoryl but no

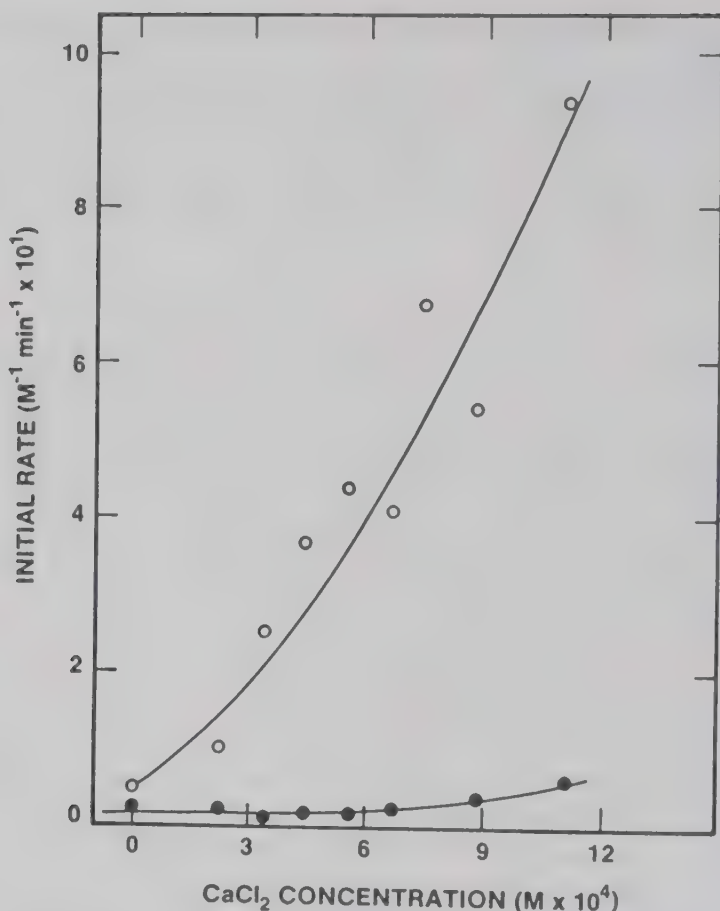


FIG. 12.8. Effect of CaCl_2 concentration on the initial rate of β -elimination and rate of addition reactions of phosvitin. The reactions were performed at 60°C in 0.123 N NaOH at $5.54 \times 10^{-6}\text{ M}$ phosvitin. The initial rate of β -elimination is expressed as $[\text{dehydroalanine}]/([\text{phosphoserine}]_0 \text{ min})$. The rate of addition to protein nucleophiles is expressed as $[\text{dehydroalanine}]/([\text{dehydroalanine}]_{\text{max}} \text{ min})$: β -elimination, $\circ$; addition, $\bullet$.

From Sen *et al.* (1977).

O-glycosyl or disulfides; and antifreeze glycoprotein containing *O*-glycosyl but no disulfides or *O*-phosphoryl.

In addition to the structure of the protein greatly influencing the reaction, in the case of *O*-phosphoryl, the rate of β -elimination is extensively increased by the presence of cations, such as calcium, apparently complexing with the phosphate (Fig. 12.8).

Early researchers such as Dakin (1912) reported that alkali-treated proteins contain D-amino acid residues. Racemization occurs as the result of the abstraction of the α hydrogen to give a carbanion inter-

mediate as shown in Eq. 3. The rates of racemization of different amino acids are dependent on the structure of the particular amino acid side chains. Masters and Friedman (1980) indicated that the rate of racemization of free aspartic acid is only 10^{-5} that of the rate of racemization of aspartic acid in some proteins. Pollock *et al.* (1979) compared the rates of racemization of amino acid residues in the same proteins that were studied for comparative purposes of the effect of alkali: lysozyme, phosvitin, and antifreeze glycoprotein-8 (Table 12.2). The rates of racemization of serine and threonine in phosvitin and threonine in the antifreeze glycoprotein are lower, perhaps because of the competing β -elimination reaction in these proteins.

When the unsaturated products of β -elimination are formed, they become prime targets for nucleophiles and the subsequent addition reactions. Some possible reactions in proteins are shown in Fig. 12.9. When the dehydroalanine formed in proteins reacts with nucleophilic groups of proteins, it leads to the formation of cross-linkages in the protein, either with the thiol groups of cysteine (in some cases formed as an intermediate in β -elimination of cystine) to form lanthionine or with the amino group of lysine to form lysinoalanine. One of the few definitive examples of the effect of protein structure on the substitutions are those from K. Hasegawa's laboratory in which the formation of cross-linkages in the homologous proteins, lysozyme and α -lactalbumin, were studied (Hasegawa and Iwata 1982). Lysozyme contains

TABLE 12.2. RACEMIZATION OF AMINO ACID RESIDUES IN PROTEINS^a

Amino Acid ^b	Lysozyme ^c	Phosvitin ^d (% D-amino acid)	Antifreeze glycoprotein ^e (% D-amino acid)
Alanine	1.02	0.89	3.56
Valine	0.42	0.00	
Leucine	0.09	1.20	
<i>allo</i> -Isoleucine	0.50	0.45	
Phenylalanine	2.98	3.91	
Tyrosine	2.62	—	
Proline	0.68	1.05	0.06
Serine	30.1	2.47	
<i>allo</i> -Threonine	12.0	5.52	4.87
Aspartic acid	16.2	6.82	
Glutamic acid	2.82	1.33	
Lysine	0.96	2.61	

Source: Whitaker and Feeney (1983).

^a Determined by gas chromatography as described by Pollock *et al.* (1977).

^b Determined after hydrolysis of the alkali-treated sample in 6 N HCl for 22 hr at 110°C.

^c 3.3 mg/ml lysozyme in 0.5 N NaOH for 2.5 hr at 22°C.

^d 3.8 mg/ml phosvitin in 0.123 N NaOH for 30 min at 60°C.

^e Antifreeze glycoprotein fraction-8 at 5 mg/ml in 0.5 N NaOH for 21 hr at 22°C.

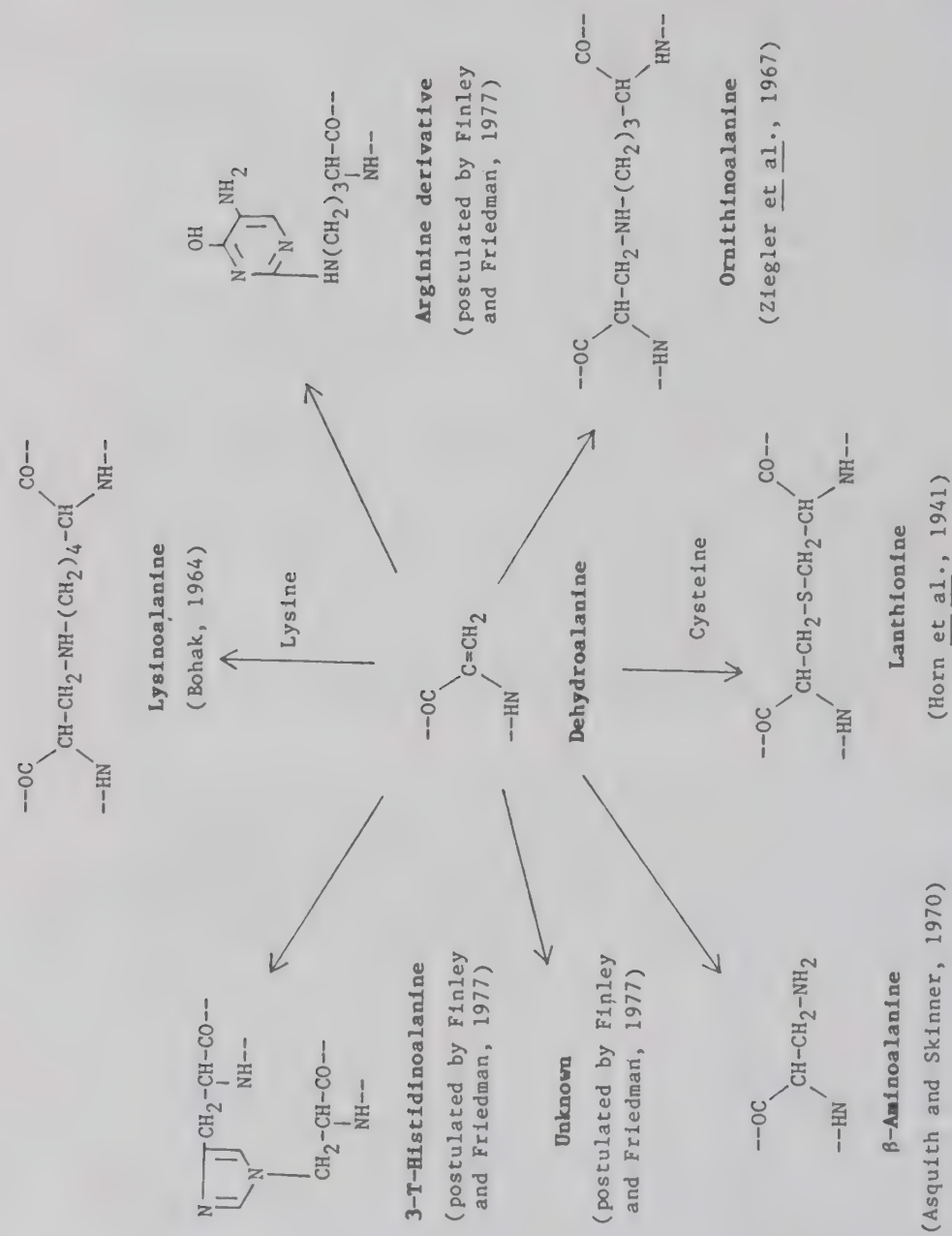


FIG. 12.9. Addition of nucleophiles to dehydroalanine residues in proteins. The arginine derivative, 3-T-histidinoalanine, and unknown derivatives which have not yet been isolated, are postulated.
 From Whitaker and Feeney (1983).

four cystine disulfide bonds, six lysine and ten arginine residues in a single polypeptide chain consisting of 129 amino acids (Canfield 1963; Canfield and Liu 1965). α -Lactalbumin contains 4 cystine disulfide bonds, 12 lysine and one arginine, and has 123 amino acids (Brew *et al.* 1970; Vanaman *et al.* 1970). More lysinoalanine formation was observed in α -lactalbumin than lysozyme, in agreement with the fact that more lysines are present in α -lactalbumin than lysozyme. A further distinct difference between lysozyme and α -lactalbumin was found in that ornithinoalanine (from arginine) formation was clearly detected in lysozyme treated at 80°, 100° and 120°C in pH 13, but not in α -lactalbumin, which contains only one arginine. In lysozyme there are three Cys-Arg and two Cys-X-Arg in the sequences. On the other hand, there is none of these in α -lactalbumin, in which no ornithinoalanine was detected.

There are, of course, many other reactions that can occur in alkaline solutions of proteins, depending upon the time, temperature, and the presence of other substances such as metal ions, sugars, and oxygen (Whitaker and Feeney 1983).

POSSIBLE UNHEALTHFUL DETERIORATIVE PRODUCTS

All food manufacturers wish to avoid any possible unhealthful products due to deteriorations on manufacturing, handling, or storage. These deteriorations appear to be under general control at almost all levels of food industry, yet the possibility is forever there because of the nature of modern techniques of processing and compounding. Certainly, one of the earlier frights was the demonstration that nitrogen trichloride (agene), which was used to bleach flour for human use, caused running fits in dogs. The product, methionine sulfoxamine (Table 12.3), was found to be the oxidized product causing the symptoms. No evidence was ever presented that agenized flour had caused similar symptoms in man, but the process using agene was stopped. Fortunately, the formation of dichlorovinylcysteine from the use of trichloroethylene for extraction of oil from soybean meal (Table 12.3) was only in products used for animal feedstuffs, but here the usage resulted in extensive economic losses due to illnesses and death of animals prior to the discontinuation of trichloroethylene as the extractant. More recently, there was concern over the presence of lysinoalanine in alkali-treated soybean proteins because of the dem-

TABLE 12.3. TOXIC COMPOUNDS PRODUCED IN FOODS AND FEEDS BY CHEMICAL MODIFICATIONS

Chemical		Use	Product	
Nitrogen trichloride (agene)	NCl_3	Bleaching flour	Methionine sulfoximine	$ \begin{array}{c} \\ \text{C}=\text{O} \\ \\ \text{HC}-\text{CH}_2-\text{CH}_2-\text{S}-\text{CH}_3 \\ \qquad \qquad \qquad \\ \text{NH} \qquad \qquad \qquad \text{NH} \\ \\ \end{array} $
Trichloroethylene	$\text{CCH}=\text{CCl}_2$	Extraction of oil from soybean	Dichlorovinyl-cysteine	$ \begin{array}{c} \\ \text{C}=\text{O} \\ \\ \text{HC}-\text{CH}_2-\text{S}-\text{CH}=\text{CCl}_2 \\ \\ \text{NH} \\ \end{array} $

Source: Feeney (1977).

onstration that such treated products containing lysinoalanine caused kidney damage in rats (Woodard and Short 1975; Nashef *et al.* 1977). No evidence was ever brought forth showing any toxicities of lysinoalanine in humans, but quality control has been strictly observed to prevent the formation of excessive amounts of lysinoalanine in foods for human use.

Yet, in spite of the lack of any evidence for harmful reactions in humans to the above three described well-defined deteriorative products, food manufacturers still must remain on the alert for possible unhealthful products. Such unhealthful products can result from the mixing of various substances in food products and subjecting them to processing conditions heretofore not studied on such a product. They could also result, of course, from a new process for which sufficient time has not been allotted for proper testing. The latter can hopefully be avoided simply by conscientiousness and prudence on the part of manufacturers. Such deteriorative products could manifest themselves directly as a toxicity, as in the use of agene (as found with dogs) and strong alkali treatment (as found in rats), from losses in nutritive value, from the formation of allergenic substances, or even from the formation of mutagenic substances.

It is well known that most all tests on food additives are done with the purified compounds and not after they have been incorporated into the food products and subjected to processing and storage conditions. Many food additives (coloring agents, flavoring agents, antiox-

idants, etc.) have reactive functional groups that are capable of forming covalent bonds with protein side chains. In addition, several are capable of being light activated and interacting in the presence of oxygen to produce oxidized products. It is not our intention here to point a finger at any particular food additives because there is a very long list and there is no evidence at this time that any cause such harmful reactions, but all, of course, could be suspect until proven otherwise. Although many are on the GRAS (generally recognized as safe) list, tests for possible covalent products with food proteins and other compounds and their possible allergenicities do not appear to have received much study. Hoseney and Faubion (1981) and Hoseney (1984) have suggested that covalent products may be formed, for example, from the addition of sulfhydryl groups to double bonds of the naturally occurring ferulic acid of wheat proteins.

Our laboratory has given a small amount of attention to the possibility of allergenic reactions. To this time only a few preliminary experiments have been done with peroxide-oxidized β -lactoglobulin. No allergenic reactions whatsoever were found with the hydrogen peroxide-oxidized β -lactoglobulin. Preliminary studies have been done on Maillard products formed by the reaction of several sugars with β -lactoglobulin. In these experiments in our laboratory (Gershwin *et al.* 1984), these products were tested for antibody class and subclass reactivity, using sera from a large sample of both normal children and children suffering from idiopathic hypersensitivity to foods. Some reactions were found to glucose-treated β -lactoglobulin. The latter observations at this time are inconsistent and need to be confirmed. They do, however, imply that there are very heterogenous reactions to antigens, even in children with the same clinical response. Thus, for example, one child with milk-associated asthma may have high-titer IgE and IgG₄ antibodies to β -lactoglobulin whereas another child with virtually the identical clinical history will not have detectable IgE or IgG₄ antibodies.

Employing a solid-phase radioimmunoassay, we analyzed the relative quantities of serum IgG₄ and IgE specific for the β -lactoglobulin in patients and controls. The role of IgE in the degranulation of mast cells and basophils is a generally accepted concept of the immediate hypersensitivity response. However, controversy exists concerning the similar function of a short-term sensitizing (S-TS) immunoglobulin of the IgG class, usually described as IgG₄ (Parish 1981; Stanworth 1983; Perelmutter 1983). IgG₄ antibody has been implicated in mediating food allergies, notably those of infants and children (Parish 1971; Pepys *et al.* 1979). Increases in the serum levels of IgG₄ have also been

observed in nonfood-related allergies (Shakib *et al.* 1977; Gwynn *et al.* 1978; Etievant *et al.* 1979; Shakib and Stanworth 1979). Human mast cells and basophils can be degraded using antibodies to IgG₄ (Nakagawa *et al.* 1981; Fagan *et al.* 1982).

It has been postulated that IgG₄ may function as a regulatory or blocking antibody (Aalberse *et al.* 1983A). It appears to be partially monovalent (Aalberse *et al.* 1983A, B) and its production is stimulated by massive (gram vs microgram amounts) and/or prolonged exposure to antigens such as foods. IgG₄ synthesis is a normal, transient response in nonatopic individuals, but is prolonged in atopics.

Sera were collected from allergic and nonatopic patients during routine examinations. These sera were obtained from Dr. D. Heiner of the Harbor-UCLA Medical Center and Dr. B. David of the L'Institut Pasteur. The sera of various laboratory personnel were also used as negative controls. Allergic patients were from 2 to 24 years of age and had milk-induced symptoms of asthma, rhinitis, and/or eczema.

Stimulation indexes (SI), the ratios of counts per minute (cpm) values of the test wells vs the values of the background controls, were calculated and compared between the groups. There was a good linear correlation between levels of IgE and IgG₄ against native β -lactoglobulin, with a correlation coefficient of 0.833. There was little correlation between the ages or sex of patients and immunoglobulin levels. However, there was much overlap in individual values between the various groups.

Our data indicate that patients allergic to milk generally have heightened levels of IgG₄ and IgE specific for β -lactoglobulin. Nonetheless individually, many have levels essentially indistinguishable from controls. Higher titers of IgE or IgG₄ immunoglobulins may be confirmatory for milk allergy, but low levels may not be useful in dismissing allergy. It has been hypothesized that chronic stimulation by tetanus toxoid or bee stings may preferentially induce IgG₄ production, while limited stimulation causes IgG₁ synthesis (Aalberse *et al.* 1983B). A similar situation may explain the low IgG₄ titers in some milk-allergic patients. This serum test has potential diagnostic value, but it cannot be fully realized until the function of IgG₄ is more clearly understood. Moreover, it was disappointing that the milk-allergic patients did not have increased sensitivity to modified β -lactoglobulin.

It is obvious that hypersensitivities to the possible reaction products from food additives in the food cannot be tested against all known food additives in all known food mixtures. Nevertheless, it would appear worthwhile to study the possible formation of chemically derived products in processed food as well as to have continual concern as to

even remote possibilities of unhealthful products. In our continuing research program, we are presently studying the possible formation of allergenic products by the interaction of proteins with the following dyes: FD & C Red No. 3, erythrosine, 3',6'-dihydroxy-2',4',5',7'-tetraiodospiro[isobenzofuran-1(3*H*),9'-(9*H*)xanthen]-3-one disodium salt; FD & C Blue No. 2, Indigo Carmine, 2-(1,3-dihydro-3-oxo-5-sulfo-2*H*-indol-2-ylidene)-2,3-dihydro-3-oxo-1*H*-indole-5-sulfonic acid disodium salt; and FD & C Yellow No. 5, tartrazine, 4,5-dihydro-5-oxo-1-(4-sulfophenyl)-4-[(4-sulfophenyl)azo]-1*H*-pyrazole-3-carboxylic acid trisodium salt.

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Some Aspects of the Chemistry of Nonenzymatic Browning (The Maillard Reaction)

*Milton S. Feather*¹

INTRODUCTION

When food preparations are heat processed or stored for long periods of time, a number of chemical reactions occur. Among these are the Maillard reaction (nonenzymatic browning). The Maillard reaction involves the reaction of an aldehyde (usually a reducing sugar) and an amine (usually a protein or amino acid) in its initial stages. The reaction, first studied and reported by Maillard (1916), is, in reality, a multiplicity of reactions. As a result of the initial interaction of sugars and amines, volatile food flavors and aromas are produced, ultraviolet (UV) absorbing compounds are produced, and dark-colored polymeric materials arise. Although this reaction is important in the processing of foods and has been the object of numerous studies, the details of the reaction are still poorly understood. Probably the most definitive (from the standpoint of chemistry) studies have involved the use of model systems, most often simple sugars and amino acids or other organic amines.

When a solution of a sugar and an amine is heated, both reactants

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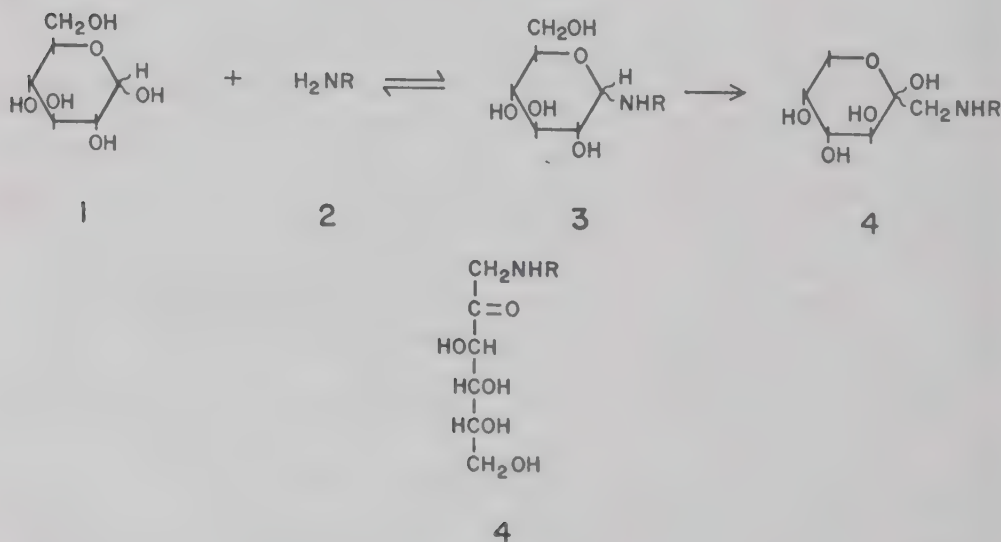
over a period of time disappear from solution, and Maillard products are formed. The purpose of this chapter is to examine the events that lead to this observation and to describe some of the materials which have been isolated.

It is noteworthy that in the past several years, several major symposia have been held on the Maillard reaction, and that the proceedings have been published. These include an international symposium, held in Göteborg, Sweden (Eriksson 1981), and an American Chemical Society symposium (Waller and Feather 1983).

SUGAR-AMINE INTERACTIONS

The Amadori Rearrangement

The initial reactions between a reducing sugar and an amine have been extensively studied and are well understood. The sugar (Scheme 1) (1) reacts with an amino group (2) to give a glycosylamine (3). This reaction is reversible. Glycosylamines, like glycosides, can be converted to the reducing sugar and the amine by hydrolysis in aqueous, acidified solution. The glycosylamine then undergoes an Amadori rearrangement to give (4), and an Amadori compound 1-amino-1-deoxy-2-ketose, shown in both the pyranose ring form and the open chain form. This portion of the reaction is essentially irreversible. The reverse reaction, known as the Heyns rearrangement, gives rise to substituted 2-amino-2-deoxyaldoses, but it appears to proceed to only a limited extent.



SCHEME 1

The formation of Amadori compounds accounts for the observed loss of both reducing sugar and amine during the Maillard reaction.

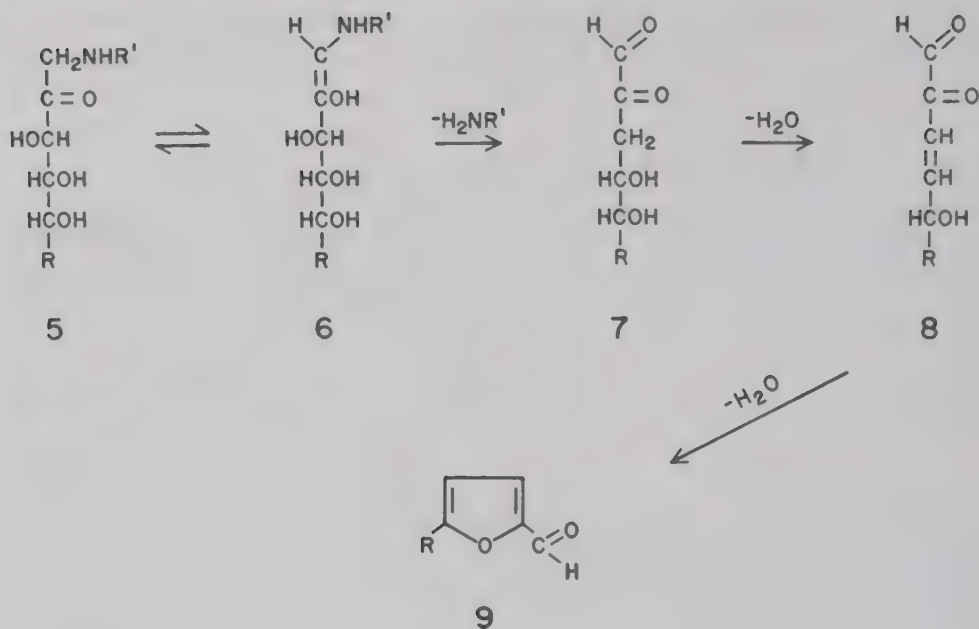
Scope and Conditions

Amadori compounds are easily produced in aqueous solutions by reacting sugar and amine in the presence of a mild acid catalyst. Yields and extent of reaction are, of course, subject to the usual rules of chemical thermodynamics and catalysis. Parameters such as pH, temperature, reactant concentration, and catalysis are all important. In model systems, using high concentrations of D-glucose and amines such as morpholine, piperidine, and toluidine, very high yields of Amadori compounds can be obtained in the crystalline state (Hodge and Fisher 1963). In more dilute solution and in foods (where the amount of reducing sugar and the availability of amino groups is low), the yields are correspondingly low, but nevertheless, Amadori compounds are detectable. Recently, a number of studies have been reported which show that the Amadori rearrangement occurs *in vivo* (Monnier and Cerami 1981) as a result of the reaction between D-glucose and hemoglobin in the blood. "Glycosylated hemoglobin" is, in reality, hemoglobin whose N-terminal end groups and certain lysyl amino groups have reacted with blood glucose to give Amadori rearrangement products, attached to the hemoglobin molecule (Bunn *et al.* 1979). Thus, the Amadori rearrangement can occur at physiological conditions (37°C, pH 7.2) if sufficient reactants are present and the reaction time is long enough (hemoglobin has a half-life in blood of about 2.5 months).

Presumably this same reaction could occur between reducing sugars and food protein. Such reactions would be expected (if substantial reaction occurs) to lead to a decreased digestibility of the protein and loss of amino acid availability. This would be expected for the cases of difunctional amino acids such as lysine, arginine, and histidine which, when present in protein, still have unreacted amino or imino groups at locations other than the α position.

Degradation of Amadori Compounds

Amadori compounds degrade via several pathways, depending on the pH of the reaction solution, the basicity of the amine attached to the sugar, and the temperature. In slightly acidic solutions (generally at pH 5.0 and lower), the degradation involves the initial 1,2 enolization of the Amadori compound (**5**, Scheme 2) to give **6**, which, on hydrolysis of the amine, gives a 3-deoxyglycosulose (**7**) intermediate



SCHEME 2

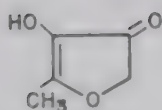
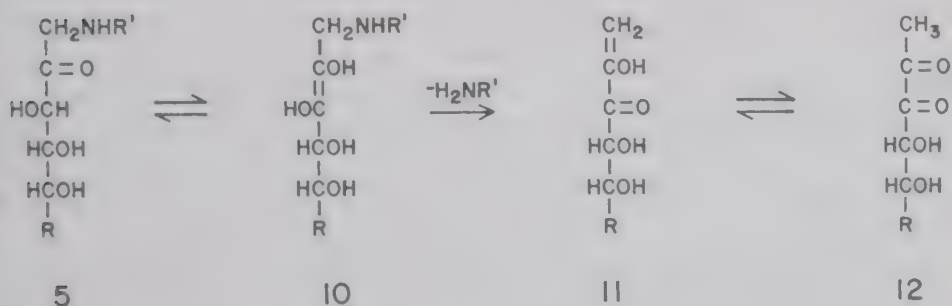
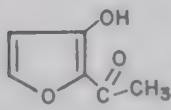
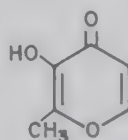
that dehydrates to **8** and thence to a furan derivative. For a hexose ($\text{R} = \text{CH}_2\text{OH}$) **9** is 5-(hydroxymethyl)-2-furaldehyde (HMF) and for pentoses ($\text{R} = \text{H}$) and hexuronic acids ($\text{R} = \text{COOH}$), it is 2-furaldehyde. Hexuronic acid derivatives decarboxylate during the reaction. A similar pathway operates for the acid-catalyzed dehydration of unsubstituted sugars as well. Intermediates **7** and **8** are thought to be common to both types of compounds. In support of this mechanism, **7** and **8** have been isolated during the degradation of Amadori compounds and have both been shown to give **9** on treatment with aqueous acid (Anet 1964).

In addition, **9** is known to be produced as a result of treating a variety of Amadori compounds with acid (Gottschalk 1952; Feather and Russell 1969). This is so for hexose derivatives (where the product is HMF), and for pentose and hexuronic acid derivatives (where the product is 2-furaldehyde). It should be pointed out that this degradation scheme is oversimplified, since **9** is produced in very low yields (usually less than 10%). Compounds **6** to **9** all represent highly reactive compounds and, in addition to leading to **9**, would be expected to undergo fragmentation and to interact with one another (or other molecules present). During a degradation reaction such as this, a variety of other low-molecular-weight compounds are produced [as evidenced by thin-layer chromatography (TLC)], and reaction solutions

rapidly darken. Eventually polymeric materials precipitate from the reaction solution. The degradation of the Amadori compound thus involves dehydration of the sugar molecule to UV absorbers such as **8** as well as the production of Maillard polymers.

In less acidic (basic) solution (pH above 5), the degradation of an Amadori compound is thought to involve 2,3 enolization to **10** (Scheme 3), followed by loss of the amine to give the enolic form (**11**) of a 1-deoxy-2,3-dicarbonyl intermediate (**12**). Compound **12** is thought to give rise to some food flavor and aroma compounds including 4-hydroxy-5-methyl-3(2*H*)-furanone (**13**) derived from pentoses and uronic acids, as well as the hexose-derived isomaltol (**14**) and maltol (**15**) (Hodge *et al.* 1972). Reaction mechanisms and parameters that control the 1,2 enolization vis-à-vis the 2,3 enolization of Amadori compounds are discussed in detail in other reviews (Anet 1964; Feather 1981). The same comments can be made relative to these types of degradations as were made about the degradation via 1,2 enolization. Compounds **13** to **15** are produced in very low yields, compounds **10** to **15** are all highly reactive, and dark-colored polymeric materials are produced during the degradation.

The fate of the amine is even less well understood. In the schemes shown, it is presumably released intact from the Amadori compound. If the amine is an amino acid, the possibility of a Strecker-type degradation cannot be excluded. This reaction involves the loss of the

**13****14****15**

SCHEME 3

carboxyl group as CO_2 gives rise to ammonia and active aldehydes containing one less carbon atom than the original amino acid. Such reactions, if they occur, would produce an even greater number of highly reactive compounds (in addition to sugar-derived intermediates) which could enter into polymerization reactions and side reactions.

MAILLARD POLYMER FORMATION

In studies using model systems (such as sugar and an aliphatic amino acid), the reactants are dissolved in a buffer and the solution heated. Discoloration occurs almost immediately (within 1 hr) and the solution turns progressively dark as a function of time. Eventually, polymeric material begins to precipitate. If aliquots are withdrawn during the reaction and suitably diluted, an intense absorption band is observed at about 285 nm. The latter is consistent with sugar-derived dehydration products being formed such as 2-furaldehydes. HMF, for example, shows a band at about 280 nm (molar absorptivity = 18,000), and a less intense band at 230 nm. Such furan derivations or degradation products similar to them may account for these absorptions. These observations lead to a number of questions such as the following: Do the UV-absorbing carbohydrate dehydration products (or reaction intermediates preceding them) play a role in polymer formation? Does the amino acid participate in polymer formation in any way? How much polymer is actually produced? Lastly (and perhaps most importantly), does a model system such as this resemble a food system in any way? Various workers have attempted to answer, in part, some of these questions, and the results are sometimes in conflict.

With respect to systems resembling actual food systems, Hashiba (1978) has reported the isolation of Amadori compounds from soy sauce preparations, and Clark and Tannenbaum (1970) have examined some pigments isolated from stored nonfat milk and casein preparations. They reported that enriched fractions of Maillard polymers (which are attached to the protein), obtained by proteolytic digestion, showed a relatively featureless UV spectrum and that elemental analysis indicated a complex mixture.

Using model systems, Velisek and Davidek (1976) studied glyoxal (a model sugar)-amino acid combinations. They report that the isolated brown polymers contain nitrogen (up to 16%). This suggests that amino acids are incorporated into the polymer. Finally, Barbetti and Chiapini (1976A) studied a D-glucose-glycine system and reported that

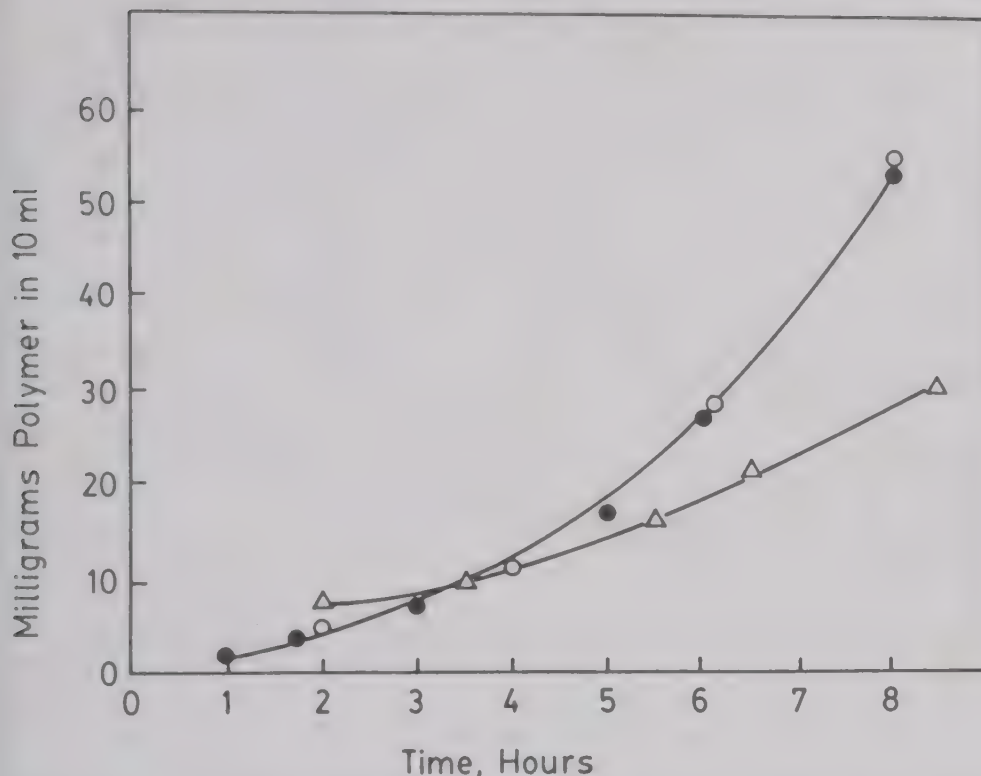


FIG. 13.1. Yields of nondialyzable Maillard polymers as a function of time using glycine as the amino acid. $\circ$, D-glucose + glycine; $\bullet$, HMF + glycine; $\triangle$, D-fructose + glycine.

these polymers may contain a furan ring (Barbetti and Chiapini 1976B) and that they show a UV absorption in the 280–300 nm range. This latter observation conflicts with a more recent report by Olsson *et al.* (1981) who concluded that such polymers show no pronounced absorption in the UV.

In this laboratory (Feather and Nelson 1984), we recently completed a preliminary study of Maillard polymers derived from simple sugars and glycine and methionine. More recently, additional amino acids have also been used.

Data on yields of nondialyzable polymer vs time were collected. These data are shown in Figs. 13.1 and 13.2. It is noteworthy that for all amino acids studied, D-fructose gave rise to lower yields of polymer than did D-glucose, although the former system darkens much more rapidly (by visual observation). On isolation by dialysis and lyophilization, the polymers were found to be water soluble. This was so for reaction times of up to 8 hr. At longer reaction times, the materials

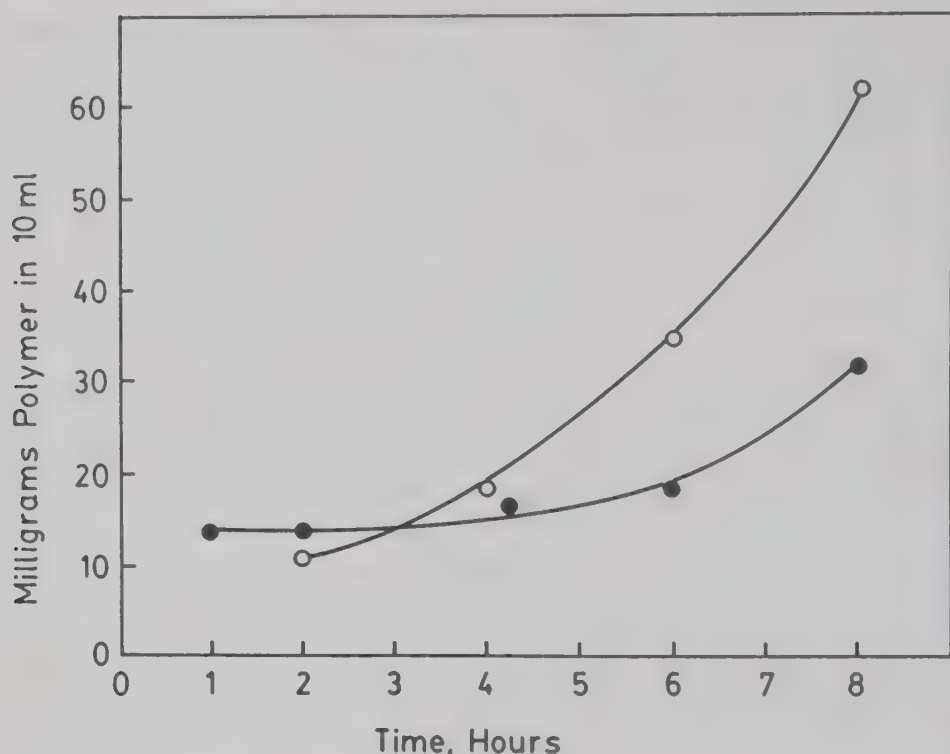


FIG. 13.2. Yields of nondialyzable Maillard polymers as a function of time using methionine as the amino acid. ○, D-glucose + methionine; ●, D-fructose + methionine.

became, as expected, progressively less soluble. Figure 13.3 shows typical UV spectra for (A) a reaction solution and (B) a polymer isolated from it. In every case studied thus far, similar data have been obtained. The absence of a UV maxima for the polymers supports the data already reported by Clark and Tannenbaum (1970) and by Olson *et al.* (1981). It is noteworthy that the polymer prepared from glycine and HMF shows no maxima. HMF absorbs UV at 230 and 280 nm intensely. Presumably during the reaction, the furan ring of HMF is opened or the conjugated system is in some way destroyed.

Elemental analyses (Table 13.1) for polymers isolated by dialysis

TABLE 13.1. ELEMENTAL ANALYSES OF SOME MAILLARD POLYMERS

Reactants	% C	% H	% N	% S	Ash
D-Glucose + glycine	51.20	5.28	6.24	—	2.91
D-Fructose + glycine	50.48	4.87	6.78	—	—
HMF(9) + glycine	47.63	3.72	3.53	—	7.63
D-Glucose + methionine	42.24	4.35	3.30	4.66	16.30

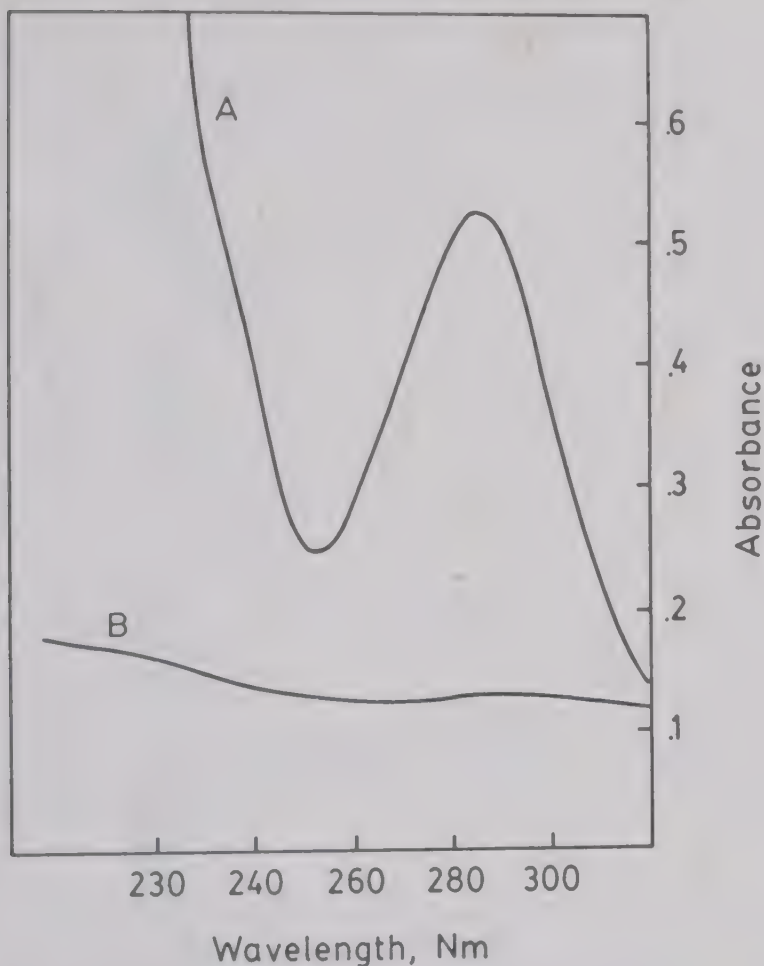


FIG. 13.3. Ultraviolet absorption spectra for (A) a reaction solution of D-glucose and glycine, initially 0.2 M for each reactant (diluted 1:2000 at 8 hr reaction time), and (B) a Maillard polymer produced at the same conditions.

against tap water show that substantial nitrogen is present as well as substantial ash. These data suggest that the Maillard polymers are charged and that the amino acid is, at least in part, incorporated. For the polymers derived from either D-glucose or D-fructose, the analyses suggest that the polymer is composed of sugar plus amino acid minus about 3 mol of water. The material derived from HMF appears to be compositionally different.

To examine some of the general chemical features of the polymer, ^{13}C nuclear magnetic resonance (NMR) spectroscopy was used as a probe. Figure 13.4 shows a natural abundance spectrum of a D-glucose-glycine polymer. It is similar to one reported by Olsson *et al.*

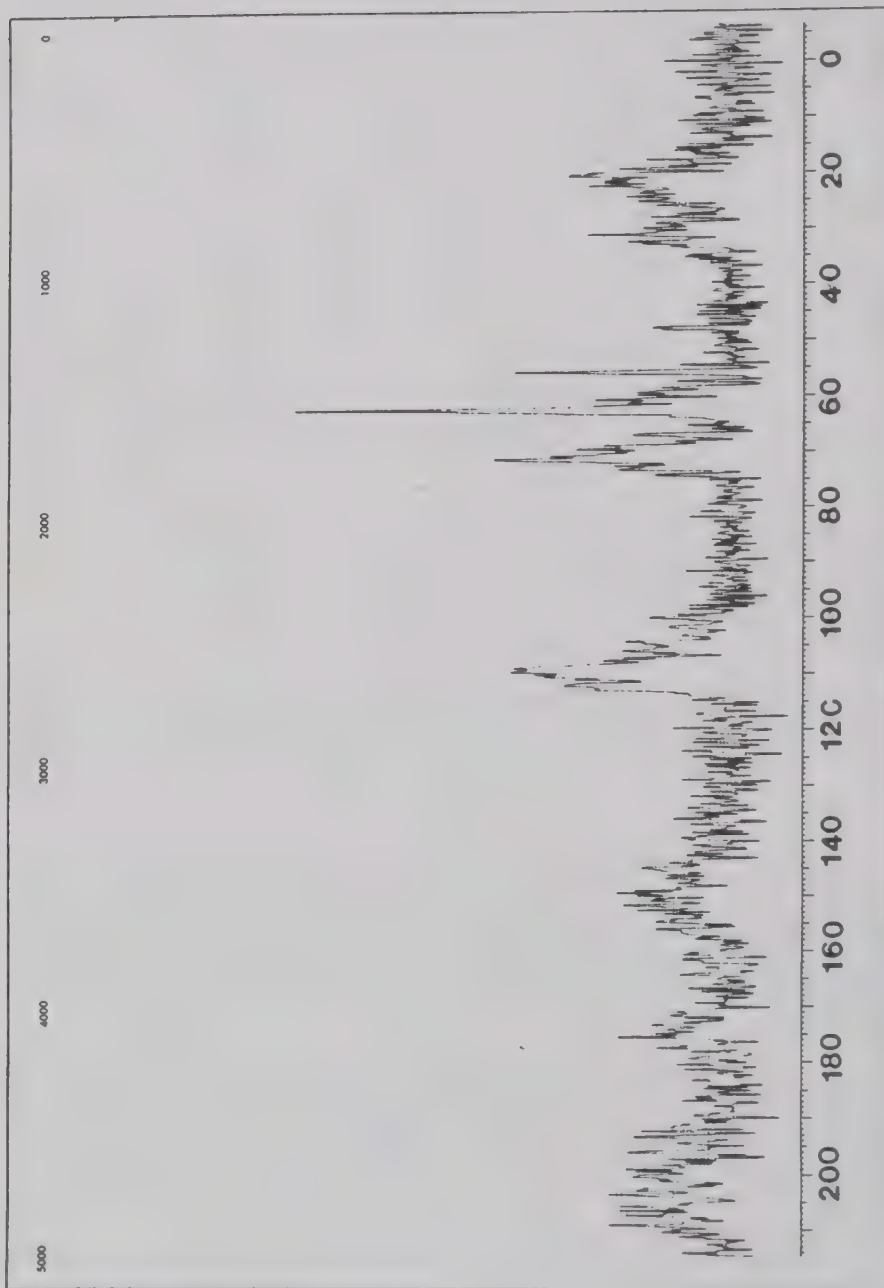


FIG. 13.4. A natural abundance ^{13}C NMR spectrum of a Maillard polymer derived from D-glucose and glycine.

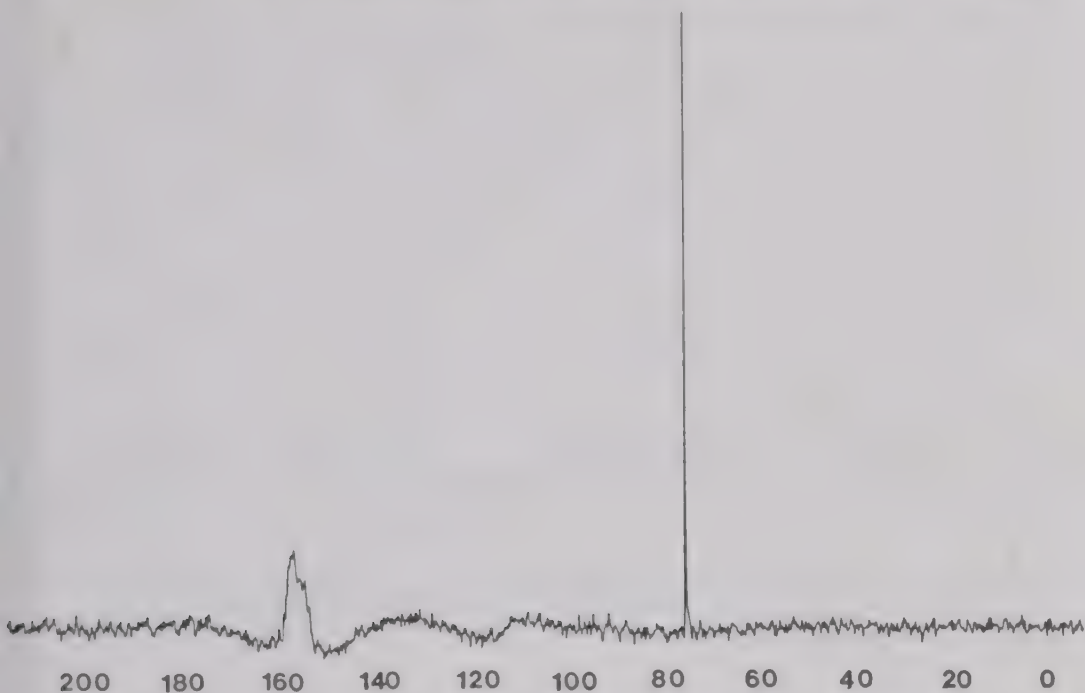


FIG. 13.5. A ^{13}C NMR spectrum of a Maillard polymer prepared from D-glucose and glycine-1- ^{13}C (90 at.%). The sharp signal at 67.2 ppm represents dioxane, used as an internal standard.

(1981) earlier. The spectra suggest that the polymer contains carboxyl (or carbonyl) carbons, as evidenced by signals in the 140–160 ppm region, as well as aliphatic carbons as evidenced by signals in the 50–80 ppm region. Little or no aromatic signals are evident further down field from the carboxyl signals.

To obtain more definitive information, polymers were prepared using glycine-1- ^{13}C (90 at.%) (Fig. 13.5) and glycine-2- ^{13}C (90 at.%) (Fig. 13.6), and NMR spectra were run. For the material prepared from glycine-1- ^{13}C (Fig. 13.5), the spectra clearly show the presence of a carboxyl (or carbonyl) signal at about 115 ppm, and for the polymer prepared from glycine-2- ^{13}C (Fig. 13.6), the signal at about 60 ppm suggests the presence of a substituted methyl group. It thus appears that glycine is at least in part incorporated into the polymer and that the chemical environment of the carbon atoms is unchanged, i.e., C-1 appears as a carboxyl carbon and C-2 as a substituted methyl group. The data do not allow quantitative interpretation, thus it cannot be said with certainty whether a percentage of the carbon atoms are lost during the reaction. In his original experiments, Maillard reported

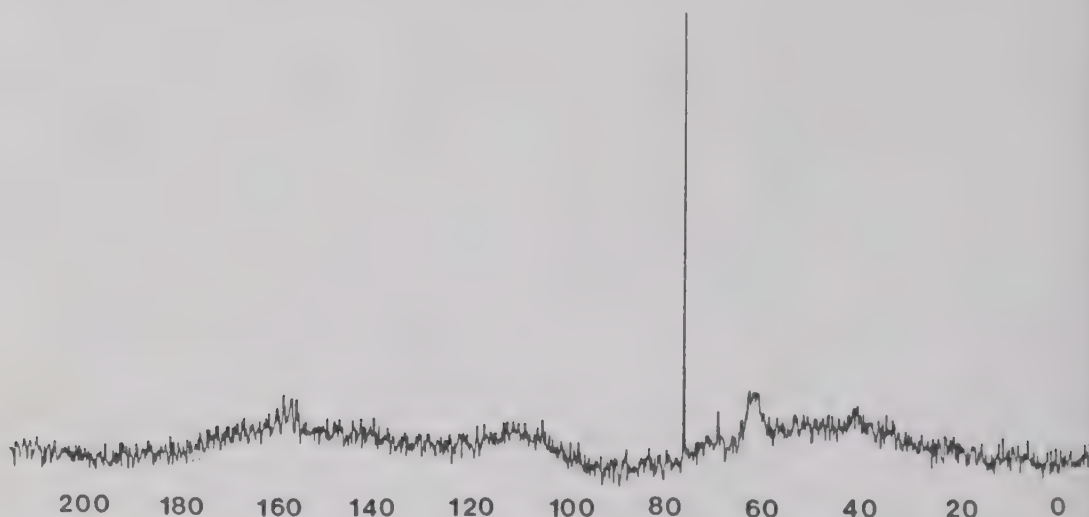


FIG. 13.6. A ^{13}C NMR spectrum of a Maillard polymer prepared from D-glucose and glycine-2- ^{13}C (90 at.%). The sharp signal at 67.2 ppm represents dioxane, used as an internal standard.

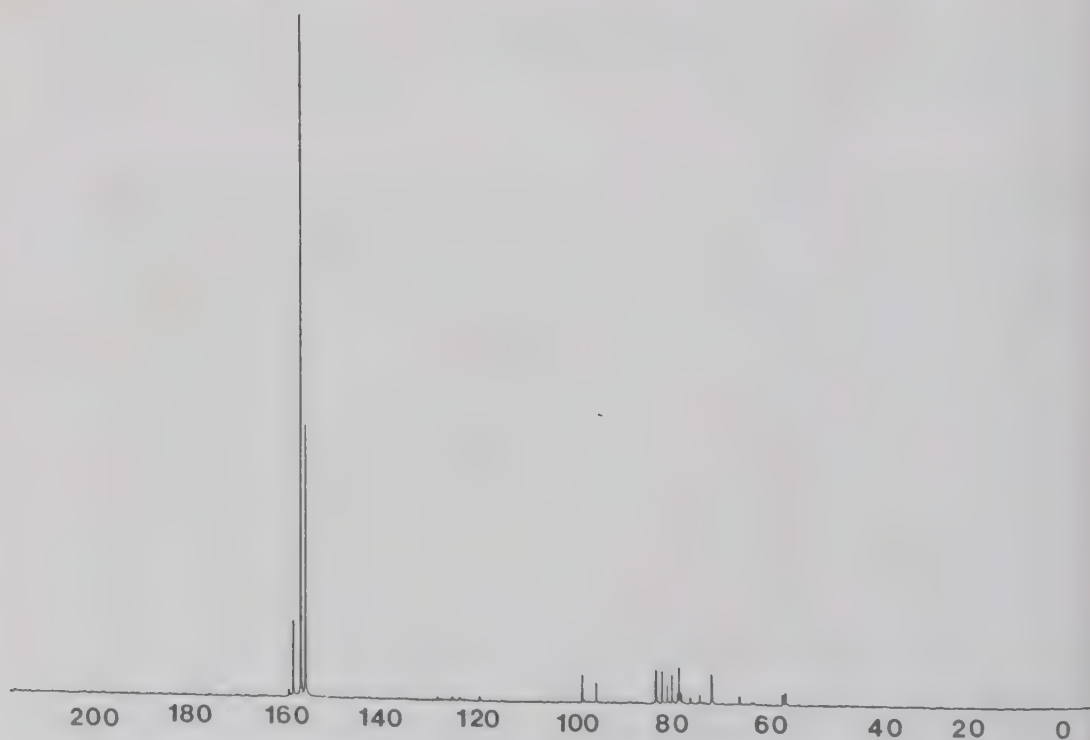


FIG. 13.7. A ^{13}C NMR spectrum of the dialyzable materials resulting from a reaction of D-glucose and glycine-1- ^{13}C (90 at.%).

the evolution of carbon dioxide. The carboxyl group of glycine would be a likely source of this molecule.

The dialyzable material, which would be expected to be low in molecular weight, was also collected, concentrated, and examined. The material produced in the glycine-1- ^{13}C reaction (Fig. 13.7) shows only three major signals (for material which contains the original C-1 of glycine). The signal at 173.2 ppm corresponds to C-1 of unreacted glycine (Stothers 1972). The other two signals (at 172.1 and 170.8 ppm) are assigned to an Amadori compound derived from D-glucose and glycine-1- ^{13}C . These assignments are consistent with assignments made by Kraska *et al.* (1975) and Funcke and Klemer (1976) for similar Amadori compounds. Two signals are observed because two forms of Amadori compounds exist in solution, namely, the β -pyranose and α -furanose forms. Based on signal intensity, the largest signal (172.1 ppm) could be assigned to the more abundant β -pyranose anomer and that at 170.8 ppm to the α -furanose anomer.

The dialyzable material derived from the glycine-2- ^{13}C experiments (Fig. 13.8) supports this conclusion. The signal at 44.4 ppm is as-

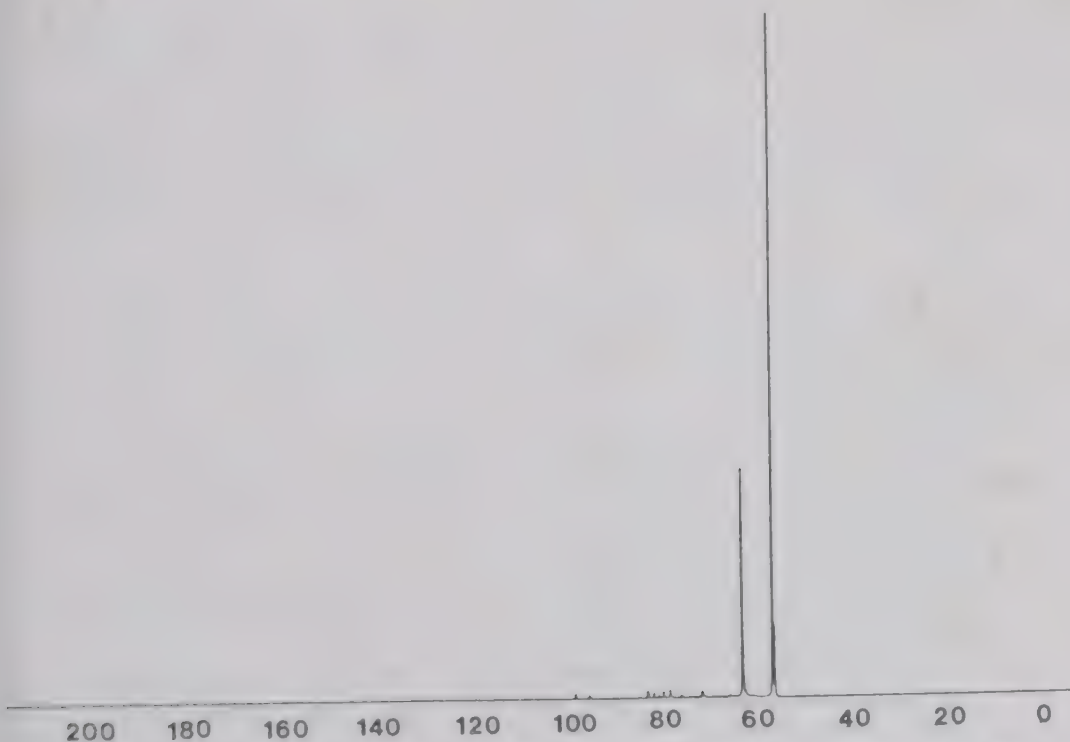


FIG. 13.8. A ^{13}C NMR spectrum of the dialyzable materials resulting from a reaction of D-glucose and glycine-2- ^{13}C (90 at.%).

signed to C-2 of unreacted glycine, and the two remaining ones at 49.7 and 40.8 ppm can be assigned to 1-deoxy-1-*N*-glycino-(2-¹³C)-D-fructose derivatives (Amadori compounds).

Although a much more complex mixture of compounds was expected, this was not observed. It appears that the major dialyzable reaction products are unreacted glycine and Amadori compounds derived from it.

The results obtained thus far for this system suggest that the polymerization process involves both sugar and amino acid, that Amadori compounds are probable precursors, that dehydration of the sugar is involved, and that, at least in part, both carbon atoms of glycine are incorporated into the polymer.

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Principal Changes in Starches during Food Processing

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INTRODUCTION

Starch, the main supplier of dietary calories to the world's human population, has many chemical and physical characteristics that set it apart from all other food components and even all other carbohydrates. It is important that food processors take note of the structural and behavioral characteristics of starch and develop an understanding of its unique properties so as to avoid food processing difficulties and to take advantage of its numerous beneficial qualities. The chemistry and technology of starch has been reviewed recently in great detail (Whistler *et al.* 1984).

Starch, unlike all other carbohydrates and edible polymers, occurs in the form of discrete particles, the starch granules. Starch granules are unique to the plant source and, hence, starch from every plant type is different in appearance and behavior from the starch of another plant type. Shape and size of starch granules are so varied between plant types that even amateur microscopists can readily identify granules of common crops.

Molecules formed by starch synthetase nucleate at points called hilums. Starch molecules are continually produced and deposited around a hilum in a generally radial arrangement to produce a spherocrystal

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likeness with a cleft at the hilum, which is generally off center. Granules have no constructed surface membrane, although residual synthetase molecules cling rather tenaciously to the surface which otherwise consists of end units of the many starch molecules laid together during the growth of the spherocrystal. Linear amylose molecules occur somewhat segregated, though not entirely so, among branched amylopectin molecules. Linear amylose molecules tend to be helical and even double helical. Branched amylopectin molecules are generally most numerous, constituting on the average about 75% of the usual granular carbohydrate. While the structure of the amylopectin molecules is variously believed to be bushlike, there is an increasing number of investigators who believe, as Ziro Nikuni (Nikuni *et al.* 1969; Nikuni 1978) originally proposed and Dexter French (1972, 1984) asserted, that the molecules have what may be called a tassel-on-a-string

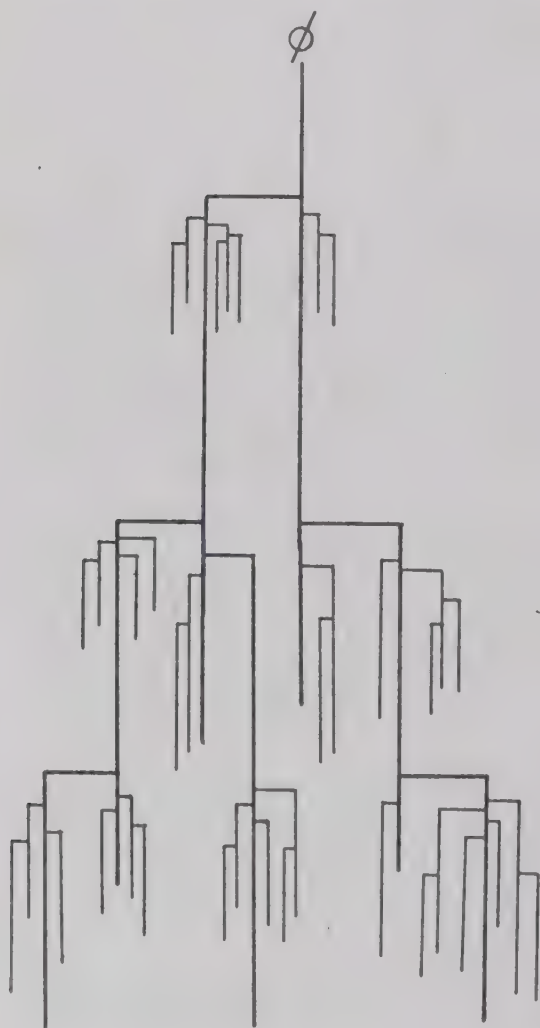


FIG. 14.1. Proposed tassel-on-a-string structure of amylopectin.

string structure (Fig. 14.1). Here short chains of 12–15 D-glucopyranosyl units occur about every 25 units of the main chain, which consists of over a thousand units. Some evidence exists that even an amylose molecule may have two to five branches in a length of 1000 D-glucopyranosyl units. If there are branches, they must consist of linear chains and must be so far apart as to allow amylose to act as an essentially linear molecule, as it can form films and be spun into fibers of strengths equal to cellulose. However, the tendency of amylose molecules to helicise gives the amylose greater elasticity than possessed by the very inelastic cellulose.

The spherocrystalline radial arrangement of starch molecules in a granule is evident from the Maltese cross, or cross of isocline, seen in polarized light in a microscope. The center of the cross is at the granular hilum. Crystallinity demonstrated in X-ray patterns shows cereal starches evidencing an A pattern that indicates chains of amylose in antiparallel double helices separated by interstitial water. Tuber and root starches produce B patterns with up to 30% water present in sheets and columns. These patterns are formed from uncomplexed amylose molecules or long lengths of otherwise structured chains. Amylopectin molecules seem to be positioned with their reducing ends inward, as would be expected from an outward biosynthetic growth of chains and branches.

Crystallinity is also evident from recorded measurements in a differential scanning calorimeter which reflects the breakup of crystalline regularity on heating and depicts the melting energy required.

Normal corn starch containing both amylose and amylopectin is biosynthesized by two enzymes, a chain-lengthening enzyme, perhaps the same enzyme for both amylose and amylopectin chains, and a branching enzyme that gives rise to amylopectin derived from a linear chain. Some evidence suggests that at least one branching enzyme requires an amylose chain of 35–40 units before it can transfer a portion of the chain to form a branch on another molecule.

Starch granules under normal microscopic examination and electron microscopic examination show lamella or growth rings around the hilum which may become wider in the outer layers as the granule grows. Some evidence points to the beginning of amylopectin molecules at the inner lamella edge and extending to the outward lamella boundary.

GELATINIZATION

Seldom is starch used in food products where most of it is retained in a granular state. For one thing, the raw cereal flavor of grain

starches is usually not desired and is lost by gelatinization. More importantly, the role of starch is principally either to take up water or to produce gel characteristics or viscosity effects, all of which are extensively controllable to provide desired texture qualities. Extent of starch gelatinization in baked goods strongly affects the properties of the product, its storage behavior, and its rate of digestibility though not its total metabolizability.

To some food chemists, it may be surprising that little gelatinization occurs in a number of food products. Thus, in cookies and pie crust a high proportion of wheat starch granules remains ungelatinized. In angel food cake all of the granules are gelatinized through a number of empty swollen granular sacks that are visible (Lineback and Wongsrikasem 1980). Unswollen granules are resistant to enzyme hydrolysis, but are attacked slowly and on ingestion contribute nearly the same total calories per unit of weight as swollen granules. The extent of gelatinization in a number of baked products is shown in Table 14.1.

TABLE 14.1 EXTENT OF GELATINIZATION IN BAKED PRODUCTS

Product	Measurement of gelatinization		
	Folding degree	Birefringence (loss %)	Enzyme hydrolysis (%)
Angel food cake	Very high	100	94 ± 4
White bread	High	100	96 ± 2
Cake doughnuts	Intermediate	98	93 ± 2
Cinnamon roll	Intermediate	98	75 ± 5
Pie crust	Low	50	9 ± 0.4
Sugar cookies	Low	9	4 ± 0.4

Undamaged starch granules are not soluble in cold water, but can reversibly imbibe water and swell slightly. The percentage increase in granule diameter ranges from 9.1% for normal corn starch to 22.7% for waxy starch. This swelling is reversible on drying. However, as the temperature is increased, the starch molecules vibrate so extensively that they break intermolecular bonds and allow their hydrogen bonding sites to engage more water molecules. This penetration of water and the increased separation of more and more segments of starch chains increase randomness in the general structure and decrease the number and size of crystalline regions. Continued heating in the presence of abundant water results in a complete loss of crystallinity, as judged by loss of birefringence and the change in X-ray pattern. The point at which birefringence first disappears is regarded as the gelatinization point, or gelatinization temperature. It usually occurs over a narrow temperature range, with larger granules gela-

tinizing first and smaller granules later, although this is not a universal pattern. Other methods for measuring gelatinization involve measuring loss of turbidity, increased solubility, dye absorption, enzyme action, chemical reactivity, changes in X-ray pattern, or changes in nuclear magnetic resonance. Of these methods one of the most sensitive, and one easy to measure, is the increase in extent of enzyme hydrolysis using glucoamylase or a mixture of α -amylase and glucoamylase, wherein the D-glucose produced is determined by glucose oxidase.

In normal commercial food processing, starch granules quickly swell past the reversible point and, due to water molecules entering between starch chains, break interchain bonds and establish hydration layers around starch molecules. This effectively lubricates chains so that they become more and more fully separated and individually solvated. At first the entrance of water causes a tangential swelling pressure that induces granules to enlarge hundreds of times, forcing some peripheral molecules to flow together to reestablish intermolecular bonding in such a way as to produce a rather delicate outer membrane. In a gently stirred and heated 5% starch suspension, granules imbibe water until almost all of it is within the granules, forcing them to press against each other and fill the container to produce a highly viscous gel or starch paste. The highly swollen granules are easily broken and disintegrated by stirring, causing a large decrease in viscosity.

In swelling of starch granules, the hydrated linear amylose molecules can more easily diffuse through the newly formed membrane to accumulate preferentially in the external water phase. This partial fractionation is responsible for some aspects of paste behavior, which will be discussed later.

Effects in starch swelling are seen in a Brabender Amylograph where viscosity is continuously recorded as temperature is constantly raised (Fig. 14.2). At the peak viscosity some of the granules have been broken due to stirring, and with continued stirring, more granules rupture to further reduce viscosity. On cooling, starch molecules reassociate, or "set back," to form a gel, the firmness of which depends upon how much interference occurs from other ingredients that may be present, such as fats, proteins, sugars, acids, and water.

Effect of Other Ingredients on Starch Gelatinization

In foods, water is not just a medium for reaction, but an active ingredient used to control texture and general physical and biological

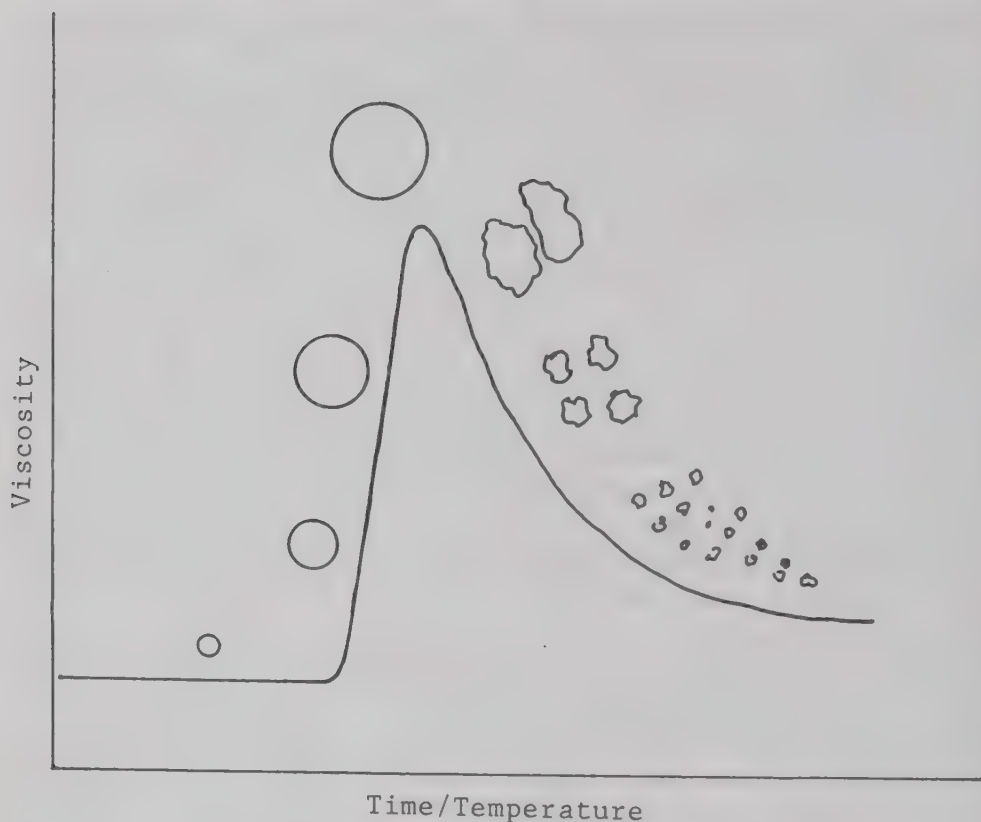


FIG. 14.2. Schematic representation of viscosity and granular changes occurring during pasting.

behavior. It is not the total amount of water that is important, but rather the availability of water, the water activity. High sugar concentrations decrease the rate of starch gelatinization, the peak viscosity, and the gel strength. Disaccharides are more effective in delaying gelatinization and in reducing peak viscosity than are monosaccharides (Osman 1967). Sugars decrease gel strength by exerting a plasticizing action and interference with the formation of junction zones between starch molecules. Lipids, such as triacylglycerol fats and oils, and lipid materials, such as mono- and diacylglycerol emulsifiers, also affect starch gelatinization. Those that can complex with amylose retard granule swelling. In white bread, which is low in fat, 96% of the starch is fully gelatinized, as evidenced by microscopic examination or by determination of the amount of starch quickly attacked by glucoamylase. Pie crust and cookies, both high in fat and low in water, have large portions of starch ungelatinized. In systems wherein starch gelatinization does occur, added fat, in the absence of emulsifiers, will

not influence maximum viscosity, but will lower the temperature at which the maximum viscosity occurs. For example, during gelatinization of a corn starch water suspension, maximum viscosity is attained at 95°C, but in the presence of 9–12% fat, maximum viscosity occurs at 82°C.

Monoacylglycerols, whose fatty acid component has 16–18 carbon atoms, inhibit swelling and cause an increase in gelatinization temperature, an increase in the temperature to attain maximum viscosity, a decrease in the temperature of gel formation, and a decrease in gel strength. Fatty acid salts or the fatty acid component of monoacylglycerols can form inclusion complexes with helical amylose, and possibly with the longer outer chains of amylopectin (Fig. 14.3). Such complexes are not easily broken and resist entry of water in the granule. Lipid–amylose complexes also interfere with junction zone formation and thus retard staling (see below).

Due to the neutral character of starch, low concentrations of salt have little effect on gelatinization and gel formation. Exceptions are potato amylopectin, which contains some phosphate groups, and manufactured ionic starches. With these starches salts may either increase or decrease swelling, depending on conditions (Osman 1967).

Starch-thickened acidic foods usually have pH values in the range 4–7, a range that has little effect on starch swelling or gelatinization.

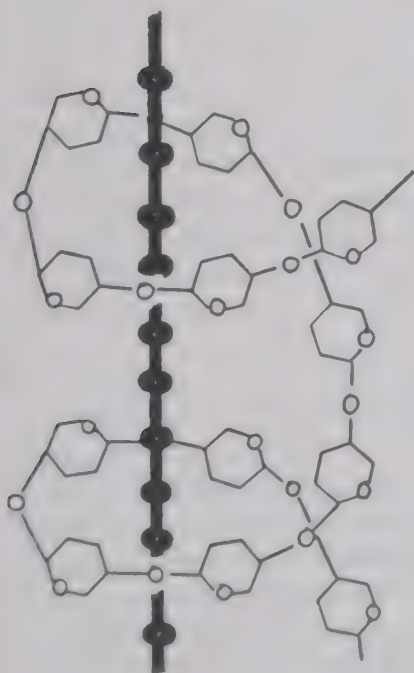


FIG. 14.3. Schematic illustration of amylose–lipid inclusion complex.

At lower pH values extensive hydrolysis occurs, yielding nonthickening dextrans. To avoid acid thinning in starch-thickened foods, a cross-linked starch is normally selected. Since cross-linked molecules are so enormous, extensive hydrolysis is required before viscosity decreases significantly.

Starch-protein interactions are important in many foods, notably batters and doughs where wheat starch and gluten interact to give structure. Gluten formation due to mixing, its denaturation, and water binding during starch gelatinization give baked goods their structure. Normal flour protein is said to add about 15% to the viscosity which would be expected if its weight percentage were replaced by starch in the flour. However, the exact nature of the interaction between starch and protein remains unclear.

Starch-thickened foods and starch gravies and pastes have poor freeze-thaw stability due mainly to retrogradation. Cherry pie filling thickened with normal starch and freeze-thawed acquires a fibrous and grainy texture. Waxy starches perform better in frozen foods than do starches containing amylose. Introduction of phosphate cross-links in starch improves its ability to function in frozen products.

Staling in starchy foods, at least in the early stages, is a consequence of association between amylose molecules. This association can be hindered by complex formation between amylose and surfactants or fatty material, including natural lipids. Staling occurring over an extended period also involves association of the longer branches of amylopectin molecules. Staling effects can be partially reversed by heating. Heat energy plus the lubrication provided by moisture allows thermal movement of starch molecules, partially restoring amorphous, looser structure that leads to a softer texture.

Starch from wheat contains up to 1% lipid consisting mainly of lysophosphatidylcholines of palmitic and linoleic acids which are mostly complexed with amylose. Such complexes hinder starch hydration and hence lower peak viscosity on gelatinization. The complexes also are more slowly hydrolyzed by amylases.

Starch Gelatinization in Foods and Its Effect on Physical Properties

Sieve analysis of low-moisture wheat toasted in air at 325°C and then cooled by water spray and rolled shows higher fines than wheat toasted at higher moisture contents, suggesting more granules gelatinized at high moisture levels (Mossman *et al.* 1973). Gelatinization at 30% moisture gives improved flake integrity because of increased gelatinization. Unswollen granules decrease from 88% in the original

wheat to 3% at the 30% moisture level. Solubles do not greatly increase, suggesting that amylose is not released in the flaking process. The percentage of damaged granules increased from less than 1% to 38% for the wheat of 30% moisture. All flaked samples showed higher maximum viscosities in Brabender measurements and maintained higher viscosity levels during the isothermal holding period, with higher setback on cooling to 50°C. Thus, in toasting and rolling, the extent of starch modification depends primarily on moisture content.

Tapioca starch gelled in skim milk at 70°C for 15 min and cooled to 4°C developed radial, peripheral channels. Some granules developed a dense, highly folded coat and a dispersed core while others developed an amorphous texture with extensive folding (Hood *et al.* 1974).

Cross-linking of starch granules gives control over degree of swelling, resistance to viscosity breakdown, freeze-thaw stability, decreased syneresis of gels, and decreased susceptibility to acid. Cross-linking of food starch is generally made with phosphate ester cross-links provided by reacting starch with controlled amounts of sodium trimetaphosphate or phosphoryl chloride. Other acceptable cross-linking agents are dicarboxylic acids, such as succinic or malonic. Depending on the cross-linking agent and its manner of reaction, cross-links can be rather uniformly distributed throughout the granule or confined primarily to the peripheral layers. By tying starch molecules together, their freedom to replace interstarch hydrogen bonds with starch-water hydrogen bonds becomes restricted due to decreased freedom for molecular separation. Such rigid covalent bonding of molecules limits swelling. Thus, cross-links can extensively limit granule swelling and can be used to control it from almost zero to almost full swelling. But even in more highly swollen granules cross-linking provides resistance to granule rupture and viscosity breakdown by mechanical effects of fluid movement, as in stirring or pumping. Because the molecules are linked together the gels produced are stronger, less fluid, and less able to increase junction zone formation and growth which would produce syneresis or the less reversible effects of freeze-thawing. Detrimental effects of freeze-thawing are a result of partial crystallization of starch molecules with attendant reduced solubility and gel clarity.

For instant dehydrated mashed potatoes, freeze-thawing reactions have been found beneficial in limiting water absorption (Ooraikul *et al.* 1974). Potatoes are peeled, sliced, steam cooked for 35 min, mashed, frozen quickly and thawed to ambient temperature, and finally dried to 6% moisture and granulated. The texture of the product is said to be excellent. Beneficial effects have been ascribed to controlled gela-

tinization, degree of retrogradation, and the amount of leached amylose which helps bind the potato cells together.

In making extruded French fries, cooked mashed potatoes were combined with predried potato starch granules, and the mixture then air dried in a fluidized bed. It was found (Jadhav *et al.* 1976) that normal potato gave less water absorbancy than when the granules were first freeze-thawed. Microscopic examination showed a thin membrane around the dried normal potato granules, but not around the freeze-thawed granules. The membrane had low solubility and appeared to be retrograded amylose which reduced water uptake. Freeze-thawed granules seemed to have a larger surface area than normal air-dried granules. Water uptake differences in the final extruded product could also be due to greater porosity of the freeze-thawed granules, since ice crystal development during freezing produces a greater porosity (Reeve 1954). The type and extent of gelatinization of starch to be freeze-thawed affect the amount of amylose leached and retrograded and hence the ability of the granules to react with other added food gums. A product containing 1.5% cross-linked phosphated corn starch, 1.5% guar gum, 0.5% high amylose corn starch, and 1% carboxymethylcellulose or methylcellulose is satisfactory to mix with mashed potato for extrusion to produce precooked French fries.

Atmospheric pressure affects rate and extent of starch gelatinization (Morrow and Lorenz, 1974). Rice starch granules at 70°C swell in water but remain intact, yet at 10,000 ft equivalent pressure the granules rupture when heated to 70°C. Water-binding capacity of potato starch increases with diminishing pressure, perhaps due to low intermolecular binding and greater porosity. Viscosity of corn, rice, and wheat starch pastes decreases when pasted at lower pressure.

Gelatinization of starch in durum wheat during spaghetti making varies with the protein content (Grzybowski and Donnelly 1977). Spaghetti samples cooked at 100°C for 5, 10, 15, or 20 min have cross sections showing gelatinization proceeding from the outside inward. Some granules remain ungelatinized after 10 min cooking, but not after 15 min, regardless of the protein content, which varied in the wheat flours from 12 to 17.6%. Higher protein wheats cooked slowly, indicative of low water penetration rates.

Cooking rates for rice can be represented by a mathematical formula involving a number of parameters, such as radius of the grain, concentration of water in the outer layers, initial water content of the grain, diffusion rate, and the amylose and amylopectin content (Suzuki *et al.* 1977). The cooking rate is principally dependent on the rate of water reaction of amylose and amylopectin at 98°C, but is

somewhat influenced by the rate of water diffusion in the cooked layer. The activation energy is near 20 kcal per mole, representing rupture of starch-starch intermolecular bonds.

An examination (Olkku *et al.* 1978) of the aqueous cooking of wheat starch with various added components reveals that high starch levels lower pasting temperature, possibly because of rapid amylose leaching. Sugars delay starch gelatinization (Bean and Yamazaki 1973; Bean *et al.* 1974) due to their hydration, thus decreasing the amount of accessible water. This lowering of water availability, or activity, can be compensated by increased temperature to restore the water activity needed for granule penetration and swelling. In this interpretation, sugars delay rather than retard gelatinization.

Wheat starch cooked alone in water produces a rapid increase in viscosity followed by a slow increase, possibly resulting from amylose release from swollen granules to provide thickening of the intergranule fluid. With wheat flour an initial rapid increase in viscosity does not occur, presumably due to the physical barrier provided by gluten on the surface of the starch granules and to a higher percentage of damaged granules in the flour produced by milling. Starch-protein interactions may also account for this rheological behavior (Cherry 1982).

Thus, it appears that in baked or heated foods, starch is a collector of moisture, and even in food products where many starch granules remain ungelatinized, starch extensively controls moisture movement and its effect on texture.

Control of starch gelatinization within cereal grains before further processing is also a way to provide control of flour behavior. Thus, micronization of corn or sorghum, wherein the grain is heated by infrared to rapidly increase temperature, brings about a degree of starch gelatinization. The grain can then be fed to animals or crushed and converted to tortilla flour. Such grains have good appearance (Johnson *et al.* 1980), flavor, and keeping quality. The increased gelatinization of starch, the amount of which depends upon the moisture content and temperature, causes the starch to be more readily hydrolyzed by enzymes and the viscosity of the flour to have a lower Brabender maximum. Tortillas made from micronized corn compare favorably with those made from commercial corn flour. Micronizing reduces mold growth and slows development of rancidity.

Pregelatinization of starch in foods generally increases ease of digestibility, although not necessarily in a linear fashion (Wootton and Chaudhry 1980). A prebake water:flour ratio of 0.45 or more is required in prebaking to produce significant gelatinization. High-moisture foods such as fruit cake and bread have higher amounts of gela-

TABLE 14.2. GELATINIZATION AND DIGESTIBILITY AS A FUNCTION OF WATER: FLOUR RATIO IN BAKED PRODUCTS

Product	Water: flour ratio (prebake)	Gelatinization (%)	Digestibility (%)
Raw wheat starch	—	2	25
Pregelatinized wheat starch	—	70	90
Shortbread	0.2	1	18
Cookie	0.2	2	25
Soda cracker	0.5	3	33
Crispbread	0.7	33	43
Wafer	1.6	40	56
Fruit cake	0.8	50	66
Bread	0.7	60	69

tinized starch granules and are more rapidly digested. Several starches and baked products show large differences in extent of gelatinization and digestibility (Table 14.2).

While pregelatinized starches are rapidly digested, they do not provide different caloric value except for high amylose starch, which is so difficult to solubilize that remaining ungelatinized granules may be unavailable for digestion. Some precooked starches give low food value to some animals because the viscosity produced immediately on eating may give satiety so that the animal eats less (Fleming 1981; Fleming and Vose 1979).

DEGRADATION

Depolymerization of starch molecules can be beneficial or detrimental depending on the properties needed in a food product. When degradation is desirable, it must be controlled to the proper level.

In the preparation of dehydrated sweet potato flakes (Walter and Purcell 1976), raw flakes are rapidly heated to 75°C and held for a time. Initially the natural α - and β -amylase react with the freshly gelatinized starch, decreasing iodine blue value and producing dextrans, maltose, and sugar. After about 10 min these effects decrease, possibly as water decreases and denaturation of enzymes occurs. Surface gelatinization and hydrolysis are extensive. Viscosity decreases with time and its effect on the reconstituted potato flake also is dependent on length of treatment. Potatoes treated with lye continue to be degraded for as long as they are heated due to alkaline hydrolysis and β -elimination reactions.

Wheat flours were processed at temperatures from 108° to 174°C (Hansen and Jones 1977) at several moisture contents. Those at 12.2%

moisture retained some unswollen granules after reaching 150°C while those at 23.8% and 33.1% had no remaining birefringent granules. The lower the flour moisture, the higher the temperature required to produce gelatinization. When gelatinization occurs water is taken up, following the common understanding that starch granules are moisture sinks which can be temperature triggered. While high temperature can force starch gelatinization and, hence, produce rapid digestibility, thermal effects can decrease protein digestibility as judged by reduced protease susceptibility.

Drum drying of flour for production of instant tortillas causes damage to about 89% of the starch in doughs subsequently made in the home compared to 71–77% damage in flours made by other more standard methods. Commercial flours have lower water absorption capacity and higher paste viscosities (Molina *et al.* 1977).

Microwave conditioning of durum wheat for semolina or spaghetti improves final product quality and yield, possibly because of changes in water distribution (Doty and Baker 1977; Watkins 1971). Starch damage is not increased by the irradiation unless the treatment is prolonged, whereupon pasting temperature decreases and rate of retrogradation increases.

Extrusion cookers can be used for making precooked corn flour to be used in the production of arepa, a thick unleavened corn cake used in Colombia and other South American countries. While cooker extruders generally produce considerable starch modification, flours used for arepa are cooked to produce minimal damage so as to increase loaf hardness caused by retrogradation and to improve shelf life (Smith *et al.* 1979). For this purpose, cookers are operated on 32% moisture dough at head temperatures of 90°–121°C. With 26–30% moisture doughs, head temperatures of 121°–135°C are needed to provide sufficient gelatinization. Properties of the final product depend upon the degree of starch gelatinization and the type of modification occurring in the extrusion process.

RETROGRADATION

Retrogradation is an attempt at crystallization by large unwieldy molecules. It is the fitting together of segments of starch chains. The initial joining of segments from two chains is called junction zone formation. Hydrated starch molecules translate segmentally by thermal energy and, in so doing, collide and replace intermolecular starch–water hydrogen bonds with starch–starch hydrogen bonds. If the length of the initial junction zone is sufficiently long that it is not immedi-

ately broken by thermal molecular movement, the zone, or length of intermolecular hydrogen bond attachment, may increase and become continually stronger as thermal motion moves remaining adjacent chain segments together in a zipper-like fashion. To this uniform junction zone, segments from other neighboring starch molecules may intermolecularly bond in like fashion to build a perfectly regular, crystalline arrangement eventually detectable by X-rays or polarized light, and, of course, being insoluble and having reduced penetrability, the crystallites are not hydrolyzed by enzymes. This reduced attack of enzymes occurs even with minute crystallization, or retrogradation, and reduced enzyme hydrolysis is the most sensitive test for the presence of retrogradation. (The binding of molecules together produces a gel or a cross-bonded structure where the junction zones or the more elaborate crystallites join starch molecules together in a large, increasingly stiff, inflexible, and insoluble structure that is undesirable in most foods. The growing intermolecular association may squeeze out water, producing syneresis.)

Freeze-thaw cycles provide repeated opportunity for junction zone formation or their enlargement to give rise to crystalline growth. Certain modified starches are made to resist this problem in freeze-thawing. These resistant starches have implanted structural irregularities such as phosphate ester or acetic ester groups. These irregularities reduce the fit between starch chains and eliminate junction zone formation which gives stability through freeze-thaw cycles. If not properly made, modified starches may thicken, become firm, and show syneresis when held at intermediate temperatures or when put through a number of freeze-thaw cycles.

Starch Retrogradation in Foods, Syneresis, and Effect on Physical Properties

Tapioca-milk gels stored at -37°C synerese and develop a grainy appearance after 60 days of storage (Hood and Seifried 1974). Heat shock in an automatic defrost freezer also can increase graininess and decrease viscosity. Starch granules and gelatinized granular sacks do not change on freeze-thawing, but their contents continually leach out and affect the exterior liquid phase. Since amylose, the prime retrograding material, leaches out first, its effect on paste or gel structure is paramount. Water-holding ability decreases as retrogradation ensues.

Corn grits that are freeze-thawed (Fennema 1966; Schoch 1968; Tressler *et al.* 1968) show severe retrogradation. Unmodified starch is changed by freezing to a microsp sponge structure with decreased water-

holding capacity and consequent syneresis on thawing. Amylose is mostly involved, but amylopectin participates to an increasing degree as products age.

Elastic modulus can be used as a measurement of retrogradation in tapioca starch and wheat starch gels as it is a linear measurement of crystallization (Kim *et al.* 1976). Tapioca starch retrogrades more slowly than wheat starch (Prentice *et al.* 1954). The low rate of tapioca retrogradation may be due, in part, to its low content of amylose (18%). Application of the Avrami equation shows, as expected, a negative coefficient for retrogradation and temperature, since crystallization occurs more slowly at increased temperature. Nucleation is spontaneous in all gelatinized starches if they are at temperatures where junction zones can form.

Cooked rice hardens on storage unless an acid such as acetic acid is present (Mitsuda and Nakajima 1977). Low pH also retards browning and bacterial growth. Some starch hydrolysis likely occurs to produce the low rate of retrogradation observed.

Physical and chemical changes in dehydrated potato granules during cold storage and freeze-thaw cycles produce retrogradation increases from 91 to 97% compared to 68–83% in normal granules (Ooraiikul and Moledina 1981). Release of water due to retrogradation occurs during storage. Rehydration rate of dehydrated potato granules decreases on storage, making these granules desirable for manufacture of extruded French fries and similar products, because the granules remain cohesive during the frying process. Retrogradation can be simulated by addition of a calcium salt to form ionic cross-links with the phosphate groups of potato starch that tend to stabilize the structure.

BREAD STALING

Theory and Measurement

While staling of baked goods is a consequence of retrogradation, it has been defined as a "term which indicates decreasing consumer acceptance" (Bechtel *et al.* 1953). Changes occur in taste, aroma, crumb firmness, crumb opacity, crumbliness, water-binding capacity, solubility, and enzyme availability. These changes also contribute to dryness and crispness. Elasticity and softness change to leatheriness and hardness. Staling costs the baking industry a huge amount of money and greatly affects customer affiliation. It is estimated that return of stale baked goods is about 8%, equivalent to 110 million pounds in 1974 (Maga 1975).

Staling can be variously measured by compressibility, stress-strain curves, force to drive a plunger a prescribed distance, and by various novel strain measurements, including Young's modulus (Cornford *et al.* 1964). The standard American Association of Cereal Chemists procedures (AACC 1969) include feel of the crumb, taste, mouth feel, firmness, flavor, and texture. Differential thermal analysis is widely applied, although there are some problems in precise interpretations. Stale bread shows an endothermic peak as do other cereal products (Axford and Colwell 1967), with the area under the curve giving a measure of the degree of staling. Similar measurements (McIver *et al.* 1968) show that gel aging with its attendant retrogradation is an exothermic reaction, since crystallization is occurring.

Differential scanning calorimetry has been more recently applied to the measurement of retrogradation. Staling in bread shows expected changes as depicted in Fig. 14.4 (Fearn and Russell 1982).

Data from this type of investigation suggest that ΔH_1 is +2.5 to +3.0 cal/g of dry starch and that ΔH_2 is -1.5 to -2.0 cal/g of dry starch. When the data are subjected to Avrami analysis, the Avrami exponent, n , is 0.75, while $n=1.0$ is obtained in stress-strain analysis. Crumb compressibility data analyzed with $n=1$ have been interpreted to mean that the Avrami rate constant is independent of specific loaf volume. In crumb modulus analysis, however, the rate constant appears to depend on loaf volume.

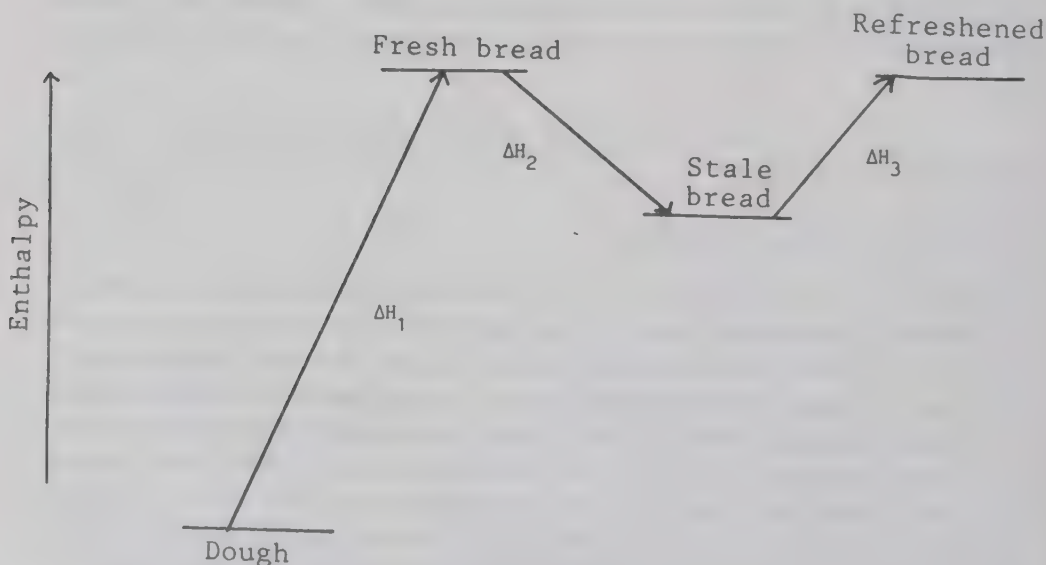


FIG. 14.4. Enthalpy changes during bread baking and staling.

In staling, the Brabender peak of bread is rapidly lowered in 24 hr (Banecki 1972) and more slowly thereafter.

Factors Affecting Staling

Some workers believe that protein-starch interaction also contributes to staling (Knyaginichev 1965), but identification of specific interactions is lacking. Some firming is possible from moisture movement from gluten, but again definitive data are lacking.

Staling of bread increases in rate as the temperature is lowered to about -2°C and then decreases at lower temperatures (Katz 1928; Cornford *et al.* 1964). At -22°C no staling occurs. Firming of bread continues at different rates under different conditions, but seems to stop at about the same firmness level. While staling is due to retrogradation, the rates are not only temperature dependent, but dependent on many other factors such as the presence of lipids, salts, sugars, and moisture. The molecular kinetics for such polymer phase change have been described by Avrami (1939, 1940, 1941) and further elaborated by others (Cornford *et al.* 1964; Luyet 1965; Kim and D'Appolonia 1977A, B). Starch crystallization appears to occur with nucleation followed by a rod-like crystal growth. Staling during the first 24 hr appears to be more complex than simple retrogradation, possibly involving protein associations and moisture movement (Willhoft 1971; Axford and Colwell 1967; McIver *et al.* 1968; Colwell *et al.* 1969; Fearn and Russell 1982). X-Ray analysis provides evidence for a similar complex mechanism (Wright 1971).

Staling proceeds less rapidly at higher moisture levels (Bechtel and Meisner 1954; Maleki *et al.* 1980). Though high-moisture breads are initially softer and remain softer than low-moisture breads, their final (72 hr) firmness is nearly the same as other breads.

Freshly baked bread of 38% moisture has crust moisture of 12% and crumb moisture of 44–45%. After storage at 21°C for 96 hr the crust moisture rises to 28% and the crumb moisture decreases in compensation.

High-protein breads may stale in stages, with retrogradation being initially prominent and protein hardening being more evident on longer storage. Work is needed to further understand moisture transfer to and from protein and the effect of protein on bread firmness. Increasing evidence suggests that protein does not alter starch retrogradation or its contribution to staling. However, high-protein breads have low overall staling rates (Kim and D'Appolonia 1977A, B).

The water-soluble and insoluble hemicelluloses of flours, termed incorrectly pentosans, may affect the rate of staling by interfering with

the movement of starch molecules. Hemicelluloses may also affect moisture distribution. Some evidence shows that these hemicelluloses inhibit retrogradation (Gilles *et al.* 1961), but other workers believe (Casier *et al.* 1973) that the hemicellulose effect is low, if any (Bechtel and Meisner 1954; Prentice *et al.* 1954; Kim and D'Appolonia 1977C, D).

Amylases from cereal, fungal, and bacterial sources when present in bread naturally or as additives soften crumb texture (Miller *et al.* 1953; Waldt and Mahoney 1967; Waldt 1968). This is especially true of α -amylases which effect random cleavage of starch molecules into initially rather large pieces, but with very small viscosity character as compared to natural starch molecules. Some α -amylases have a high thermal stability, are thermoduric in modern terminology, and hence remain through most or all of the baking process with maximum hydrolytic effect at high temperature, but with a continuing small effect after bread is cooled. Bacterial α -amylases are more often highly thermoduric and these, when used as additives, produce a very soft crumb (Dragsdorf and Varriano-Marston 1980). Breads containing α -amylase increase in crystallinity on storage, with the largest increase between 2 and 16 hr after baking, as judged by X-ray analysis. Bacterial α -amylase-containing bread is more crystalline than normal bread. The greater crystallinity is due, in large part, to the general linear nature of the dextrans produced by α -amylase hydrolysis. These shorter molecules derived both from linear amylose molecules and the linear branches of amylopectin molecules can be more easily organized by low thermal energies and thus more easily brought into a crystalline state.

Addition of surfactants in bread making causes little increase of crumb softness in fresh bread, but significantly decreases the rate of staling (Chung and Pomeranz 1977; Knighty 1977). Retardation of staling is a consequence of complex formation between the aliphatic chains of the surfactant and the helical amylose, and to a lesser degree the long ends of amylopectin molecules. X-rays show a V-type pattern indicative of such structures that are more numerous in breads with added fats and special surfactants. With aging, the B-type pattern, characteristic of retrograded amylose, increases in normal bread, but increases much more slowly in breads made from dough with added fats or special surfactants such as 2-*O*-stearoyl lactate, succinyl monoglyceride, or glyceryl monostearate. Certain surfactants added to doughs initially bind to gluten during dough mixing, but migrate to amylose in the finished bread (DeStefanis *et al.* 1977). Possibly the surfactant-gluten bonds are weakened by denaturation of the protein at high temperature and by the added movement or translation pro-

vided by the high temperature plus the thermal agitation of amylose to allow preferred complex formation.

Addition of water-binding agents such as pectin or glycerol shows that pectin increases water absorption while glycerol has no effect (Galal and Johnson 1976). On aging, the pectin-containing bread staled faster, perhaps because of its competitive removal of water from starch, allowing for easier starch-starch intermolecular bonds.

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Chemical Changes in Flavor Components during Processing

R. Teranishi and R. G. Buttery¹

INTRODUCTION

As the population on this earth increases, the problem of feeding all the people becomes greater. Even now millions go to sleep hungry. In order to solve some of the problems encountered in getting the food from where it is grown to where it is consumed, the raw material must be processed to make certain that the food reaches the consumers in a safe, nutritious, and palatable condition. Certainly, food must supply sufficient calories to fuel the body, and it must supply the essential nutrients to keep the body functioning in a healthy condition, but it is obvious that the most nutritious materials will be wasted if they are not consumed. Therefore, food processing studies should be accompanied by palatability studies.

All components in foods contribute somewhat to palatability because they contribute to color, hardness or softness, juiciness or dryness, taste, odor, etc. Carbohydrates, lipids, proteins, vitamins, and so on break down during processing, and their fragments combine with one another to form desirable or undesirable flavors. Some representative chemical changes that occur during processing and that affect flavor will be discussed here.

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Some flavor changes that occur during processing are to be avoided as much as possible, such as with flavors of fresh fruits and vegetables. Other flavor changes during processing are highly desirable, such as those during ripening of cheese, baking of cakes and breads, and roasting of meats. Flavor changes can be separated into those occurring at ambient temperatures due to enzymatic and/or microbial action and those occurring at higher temperatures during cooking.

ENZYMATICALLY AND/OR MICROBIALY PRODUCED COMPOUNDS

Green Fruity Aromas

Because oxidative changes in lipids are discussed in detail by others, the enzymatically induced breakdown of unsaturated fatty acids in foods will be discussed only briefly here in spite of the importance of this topic to changes in flavor during processing.

Compounds having "green" odors, such as (*Z*)-3-hexenol, hexanol, (*E*)-2-hexenal, hexanal, and (*Z*)-3-hexenal, seem to be formed from enzymatic oxidation of linoleic and linolenic acids. These compounds are found in most vegetables and fruits, especially when they are cut or crushed during processing. Tomatoes produce more than usual amounts of (*Z*)-3-hexenol and (*Z*)-3-hexenal; therefore, it is understandable why these compounds are reported by Pyne and Wick (1965) and Kazeniak and Hall (1970) as having the odor of fresh-cut tomatoes. (*E*)-2-Hexenal seems to be important in the aroma of apples (Flath *et al.* 1967) and, together with (*E*)-2-hexenol and linalool, in the aroma of blueberries (Parliment and Kolor 1975).

Apple peels and cores, especially in the United States, are a common by-product of apple processing operations in which the main product is slices or applesauce. In some plants, peels and cores are continuously chopped in a hammer mill and pressed to yield a solution that is stripped to recover aroma. Guadagni *et al.* (1971A, B) showed that if peels are held at room temperature for 1–2 days, the components associated with apple aroma increased 10- to 30-fold. Freshly cut peels have a predominant greenish odor which is attributed to (*E*)-2-hexenal. With time, the green odor decreases and is replaced with a fruity odor which is attributed to 2-methylbutyl acetate, which increased with storage. Other esters, such as ethyl acetate, ethyl propionate, ethyl 2-methylpropionate, 2-methylpropyl acetate, ethyl butyrate, butyl acetate, ethyl 2-methylbutyrate, pentyl acetate, butyl propionate, ethyl hexanoate, and hexyl acetate also increased with storage. Sensory tests verified the chemical findings and showed that peels conditioned at ambient temperatures by enzymatic development

of aroma compounds yielded essences with 2–7 times more “flavoring capacity” than essences from no-delay peels with no significant sacrifice in quality (Guadagni *et al.* 1971A, B). This is an excellent example in which processing conditions can be manipulated for optimum yield of desirable flavors by knowing what compounds contribute to certain flavors and by following the rise and fall of these compounds under various conditions.

Another example of following flavor quality of apple after harvest and during storage is the excellent work by Willaert *et al.* (1983). These workers showed that at harvest of the fruit only minor concentrations of aroma compounds are present. In the short-term storage of up to 10 days, maximum flavor is developed. In the long-term storage of weeks, even under controlled atmosphere, there is a decrease in aroma quality.

Cheesey Off-Aromas

Milk is mild and bland (Badings *et al.* 1981), but cheeses processed from milk range from mild to strong, the latter being characteristic of ripened cheeses (Day 1967). Because cheeses are composed of proteins, lipids, and carbohydrates, and in the ripening process these components are broken down by microorganisms to smaller molecules which react with one another, many compounds contribute to the complex flavors of different cheeses. Specific microorganisms yield characteristic cheeses with specific textures and flavors (Day 1967).

One of the interesting features of cheeses is the amount of free fatty acids present in them. Free fatty acids are considered as desirable in cheeses, whereas in most other foods free fatty acids are considered as undesirable and associated generally with rancidity and spoilage. Free fatty acids are found in most ripened cheeses and contribute somewhat to all cheese flavors, but Patton (1963) studied the volatile acids from Cheddar cheese, and after removing compounds of other functional groups with various derivatives, he concluded that acetic, caproic, and caprylic acids represent the backbone of Cheddar cheese aroma. Day (1967) reports that the balance between free fatty acids and other flavor constituents is more important than free fatty acids alone, but it seems undeniable that free fatty acids do play an important role in cheese flavors.

Papaya (*Carica papaya*) is consumed at present predominantly as a fresh fruit. However, with the adoption of uniform standards through a marketing order granted by the U.S. Department of Agriculture, increasing amounts of off-grade papayas have become available for processing. The problem of off-flavors has prevented the processing of

papayas to acceptable purees. Chan *et al.* (1973) have shown that purees deactivated by heat or acid treatment did not develop the off-*aroma* from the bacterial and/or enzymatic production of butyric, hexanoic, and decanoic acids and their methyl esters, as with the untreated purees. Knowledge of constituents contributing to the off-*aromas* has provided a solution for processing conditions to eliminate or greatly reduce the offending off-*aromas*. The interesting point in the case of cheese and of papaya is that the offending compounds in papaya contribute to the desirable qualities in cheese

Wine-like Aromas

There are too many flavor compounds formed by the fermentation process to produce alcoholic beverages to be discussed in detail here;

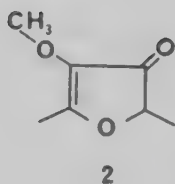
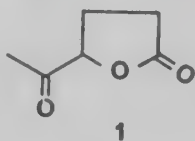


FIG. 15.1. Compounds with sherry-like aromas. (1) Solerone; (2) 2,5-dimethyl-4-methoxy-3(2*H*)-furanone.

however, solerone, reported to have a “sherry” and a pleasant wine-like aroma, must be mentioned here because it is a very important flavor compound. Augustyn *et al.* (1971) isolated and characterized 4-hydroxy-5-ketohexanoic acid γ -lactone (1, Fig. 15.1) and assigned it the trivial name of solerone because it was found in wines matured under flor in soleras. This is another excellent example of scientists being able to find the important flavor compounds in spite of the very complex mixture of compounds present. Another compound which is reported to have a sherry-like aroma is 2,5-dimethyl-4-methoxy-3(2*H*)-furanone (2, Fig. 15.1) (Hunter *et al.* 1974).

Terpenoids

There are two major ways in which terpenoids can be produced in certain food products by the conditions that occur during typical processing procedures. One involves the enzyme-initiated oxidative

breakdown of carotenoids and the other the hydrolysis of terpenoid glycosides. Oxidative breakdown of carotenoids occurs when a vegetable or fruit is broken up for processing, such as in the preparation of tomato sauce. The oxidation seems to be initiated by oxidative enzymes present in the vegetable or fruit material and may be coupled with the oxidation of the lipid, which also occurs under these conditions. In tomatoes, it was noticed that the major terpenoid compounds produced by blending of the tomatoes (6-methyl-5-hepten-2-one, geranylacetone, and farnesylacetone) were related to the structure of the main tomato carotenoid pigment, lycopene (Buttery *et al.* 1969). The more exact relationship between the particular carotenoid and the compounds formed was studied in detail by Stevens (1970) who showed that these ketones probably resulted from breakdown of lycopene, phytofluene, and phytoene, respectively (Fig. 15.2). Similarly, α - and β -ionones probably result from oxidative breakdown of α - and β -carotenes. The polyene carotenes are apparently cleaved preferably at the first conjugated diene double bonds (Stevens 1970).

In the other main type of degradation where volatile terpenoids are produced, it was noticed in some vegetables (tomatoes, bell peppers, potatoes) that there was a considerable increase in the concentrations of one or more of the terpene alcohols linalool, α -terpineol, and terpine-4-ol when the product was heated (e.g., Buttery *et al.* 1971). A related formation of monoterpenoids was also found under conditions similar to those used in processing of grape juice in the production of wines. The volatile terpenoids in processed grape juice such as linalool, α -terpineol, and geraniol seem to be derived from hydrolysis of polyhydroxylated terpenoid compounds under the normal acid conditions of the juice. This process is considerably enhanced by heating. Some of the original precursors have been identified in grapes as disaccharide glycosides (β -rutinosides, Fig. 15.3) of geraniol, nerol, and linalool (Williams *et al.* 1980, 1982). α -Terpineol can apparently be produced from these precursors, during the acid hydrolysis conditions, by ring closure and rearrangement.

Soy Proteins

Microbial transformation, or fermentation, is one of the oldest and highly acceptable methods of producing and preserving foods. (Steinkraus 1983). In the Western world, fermentation is used for alcoholic beverages, leavening bread, ripening cheeses, processing pickles and sauerkraut, etc. In the Western world, soy proteins are being used as extenders in meat products, but in the Orient, soy proteins have been consumed for thousands of years as traditional soy protein foods, fermented or nonfermented. As the world population increases, the pro-

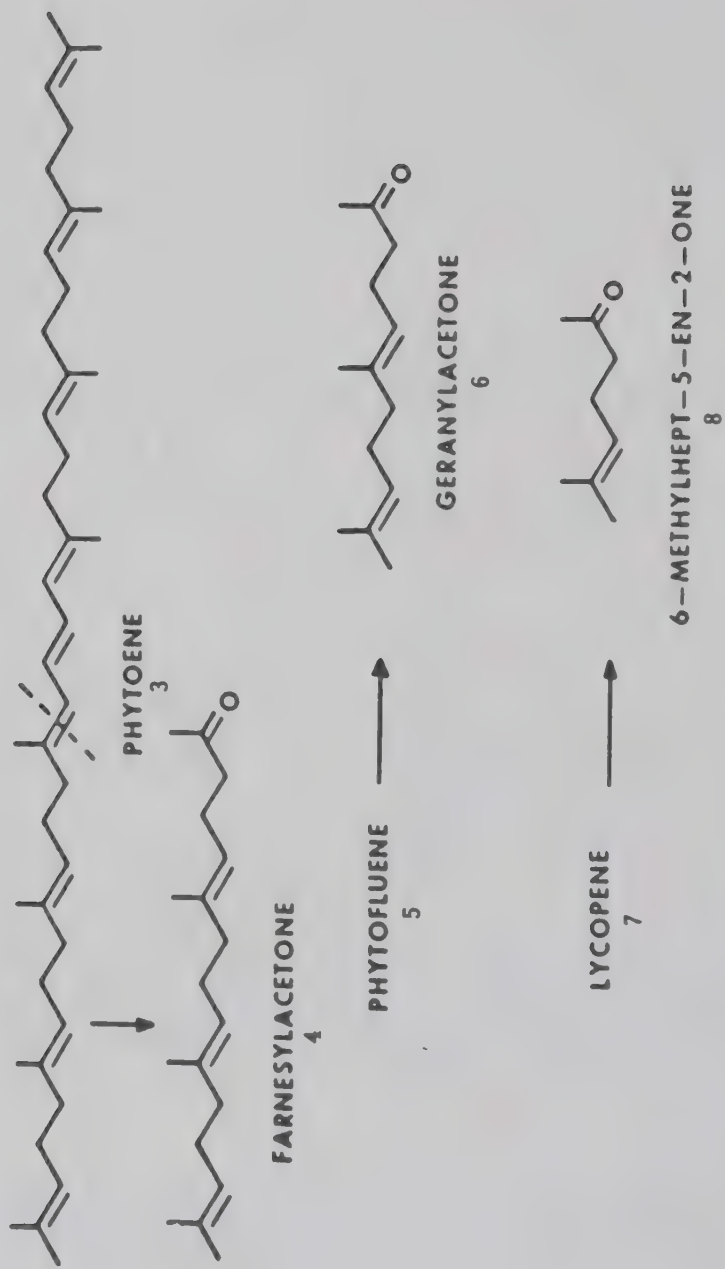


FIG. 15.2. Fragmentation of carotenoids.

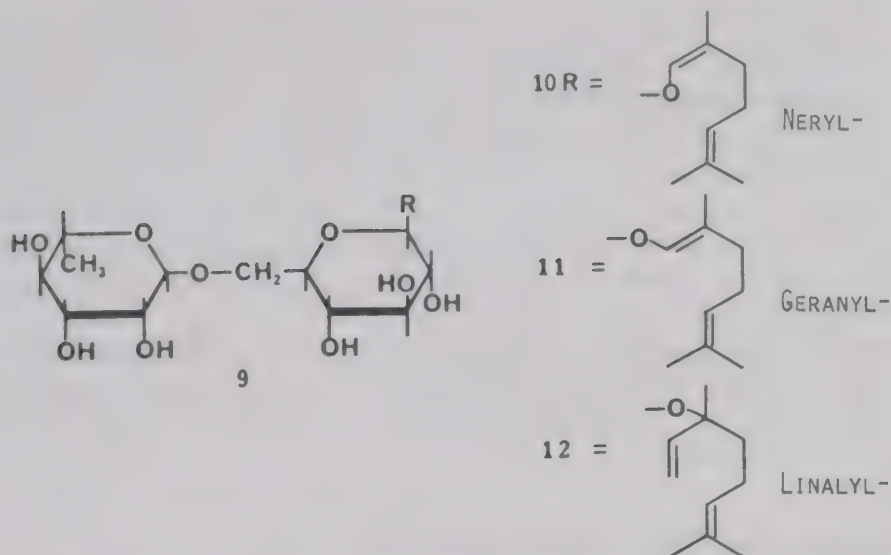


FIG. 15.3. Fragmentation of (9) β -rutinosides, (10) neryl-, (11) geranyl-, and (12) linalyl-.

duction of meat-like flavors from vegetable protein will become more important. The nonfermented soy protein products such as soy milk and soybean cake (tofu) are very bland, but fermented products such as shoyu (soy sauce), miso, natto, and tempe are very flavorful. The consumption of some of these soy protein products is increasing rapidly in the United States, especially for tofu and soy sauce. Other fermented soybean products, such as soy paste (sufu, miso, and tempe) and whole soybeans (natto), are not consumed much in the Western world, but are consumed widely in the Orient (Fukushima 1979).

In the fermentation process of converting soybeans to soy sauce, much of the starch is converted to simple sugars and proteins to small peptides and amino acids, and these smaller molecules rearrange and combine to form the complex, appetizing flavor of soy sauce (Fukushima 1979). Nunomura *et al.* (1980) have shown that of the many compounds that have been identified in soy sauce volatiles, 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone is the main and most important constituent of the characteristic flavor of soy sauce. Hydroxyfuranones are also very important in nonenzymatically formed flavors and are discussed in the section on thermally formed flavors.

THERMAL CHANGES

Although texture is not in the category of flavor, texture plays a definite role in palatability: Wilted lettuce and limp celery are not as

appetizing as crisp lettuce and crunchy celery. In processing, it should be remembered that gelling of starch occurs in the range of 70°–80°C, and that denaturation of protein occurs over a wider range than that of gelling of starch because protein occurs in a greater range of structures than starches. Although chemical changes in macromolecules are important in palatability, they will not be discussed here. This discussion will be restricted to thermal fragmentation of carbohydrates, proteins, lipids, etc., and the combination of these fragments to form compounds which contribute to flavor.

Some of the thermally induced chemical changes discussed here will be the caramel-like aromas produced by nonenzymatic browning, cracker-like compounds by roasting, and various compounds produced by cooking of meats.

Nonenzymatic Browning Compounds

One of the most delightful aromas is that of vapors wafting from bakeries, aromas of nonenzymatic browning reaction compounds from the baking of cookies, pies, cakes, breads, etc. In the process of caramelization of sugars, the color goes from colorless to pale yellow to dark brown, and finally to black, with flavor changes accompanying the color changes going from sweet to bitter and acrid. In processing, the challenge is to optimize the desirable caramel-like odors and to minimize the burnt, bitter, and acrid notes. To do so, it is helpful to know what compounds contribute to these properties and under what conditions they are formed. Products of carbohydrate caramelization and pyrolysis are listed by Hodge (1967), and flavor properties are described. Time has not diminished the value of this excellent review article by Hodge (1967). Tressl *et al.* (1979) have reviewed the chemical formation of flavor substances. A more recent review covering aroma compounds from nonenzymatic browning reactions with an excellent discussion of structure–odor relationships has been presented by Ohloff (1981).

Maltol (**13** in Fig. 15.4; see also Table 15.1) has been known for many years for its fragrant, caramel-like aroma and is used to enhance the flavor of many products (Hodge 1967). Another compound that has been known for many years is 3-methyl-2-hydroxy-2-cyclopenten-1-one (**14**), which has a sweet and licorice-like aroma (Hodge, 1967). Another very interesting compound that has a caramel-like and cooked pineapple aroma is 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (**15**), prepared by Hodge *et al.* (1963) and isolated from pineapples by Rodin *et al.* (1965).

Enough chemistry has been done to vary the structure of compound

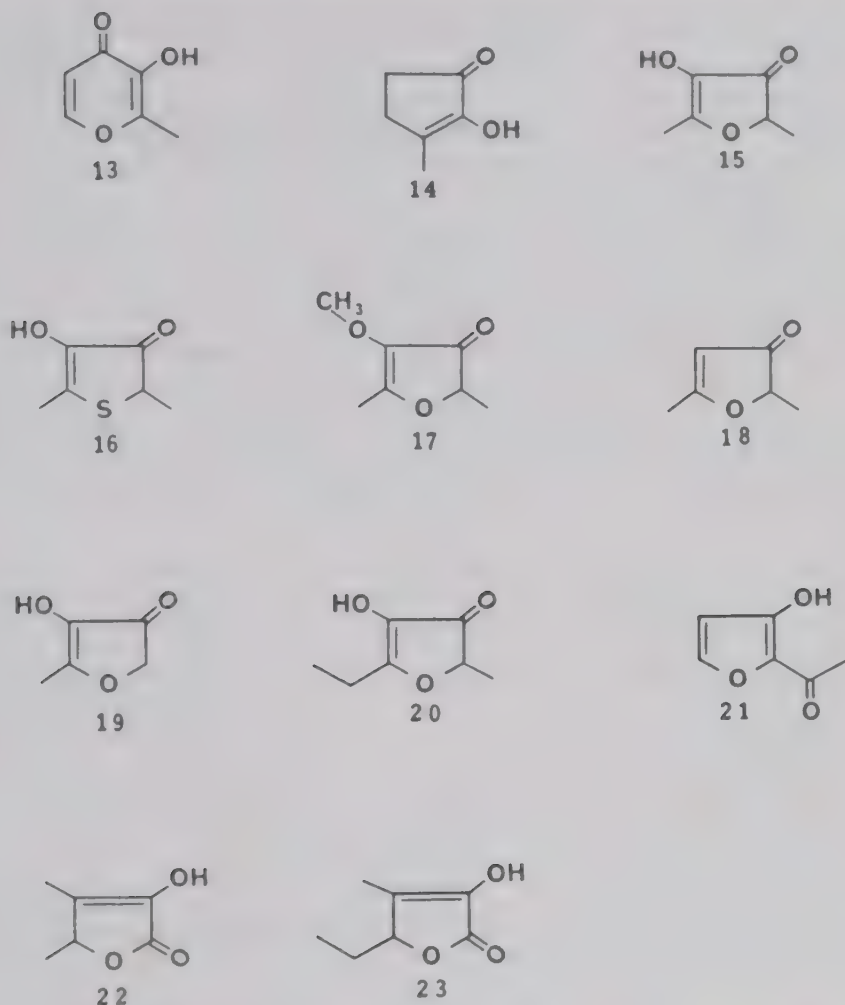


FIG. 15.4. Nonenzymatic browning compounds. Names are listed in Table 15.1.

15 so that some interesting correlations can be made with structure-odor relationships in the hydroxyfuranone series. If sulfur is substituted for the oxygen in the furan ring in 15 to form 2,5-dimethyl-4-hydroxy-3-oxo-2,3-dihydrothiophene (16), there is not much modification of the odor except for a more pronounced burnt odor (Ohloff 1981). By replacing the proton on the hydroxyl with a methyl group, 2,5-dimethyl-4-methoxy-3(2H)-furanone (17), the odor changes so that it contributes to the characteristic of Finnish wild strawberries (Pyysalo *et al.* 1979) and to that characteristic of sherry wine (Hunter *et al.* 1974). It is interesting that solerone, a lactone shown in Fig. 15.1 (Augustyn *et al.* 1971), also contributes to the sherry wine odor. When

TABLE 15.1. NONENZYMATIC BROWNING COMPOUNDS—STRUCTURE/ODOR RELATIONSHIP^a

Compound	Odor characteristic	Reference
(13) Maltol	Sweet, caramel-like	Hodge (1967)
(14) 3-Methyl-2-hydroxy-2-cyclopentene-1-one, Cyclotene (trade name)	Sweet, licorice-like	Hodge (1967)
(15) 2,5-Dimethyl-4-hydroxy-3(2 <i>H</i>)-furanone	Caramel-like, cooked pineapple	Hodge <i>et al.</i> (1963)
(16) 2,5-Dimethyl-4-hydroxy-3-oxo-2,3-dihydrothiophene	Caramel-like, slightly burnt	Ohloff (1981)
(17) 2,5-Dimethyl-4-methoxy-3(2 <i>H</i>)-furanone	Sherry wine-like, Finnish wild strawberries	Hunter <i>et al.</i> (1974) Pyysalo <i>et al.</i> (1979)
(18) 2,5-Dimethyl-3(2 <i>H</i>)-furanone	Freshly baked bread	Rosenkranz <i>et al.</i> (1963)
(19) 5-Methyl-4-hydroxy-3(2 <i>H</i>)-furanone	Caramel-like, cooked beef-like, roasted chicory root	Nunomura <i>et al.</i> 1979 Tonsbeek <i>et al.</i> (1968)
(20) 2-Methyl-5-ethyl-4-hydroxy-3(2 <i>H</i>)-furanone	Caramel-like, soy sauce-like	Nunomura <i>et al.</i> (1979) Nunomura <i>et al.</i> (1980)
(21) 2-Acetyl-3-hydroxyfuran isomaltol	Burnt sugar, fruity	Fisher and Hodge (1964) Hodge (1967)
(22) 4,5-Dimethyl-3-hydroxy-2(5 <i>H</i>)-furanone (2-hydroxy-3-methyl-2-pent-2-enolactone) (sotolon)	Sugary	Tokimoto <i>et al.</i> (1980) Rijkens and Boelens (1975) Takahashi <i>et al.</i> (1976)
(23) 4-Methyl-5-ethyl-3-hydroxy-2(5 <i>H</i>)-furanone (2-hydroxy-3-methyl- γ -hex-2-enolactone)	Sweet, curry-like, vegetable protein hydrolysate	Sulser <i>et al.</i> (1967)

^a Chemical structures of compounds are shown in Fig. 15.4 and correspond to the boldface numbers given here.

the hydroxyl is missing, as in 2,5-dimethyl-3-(2*H*)-furanone (**18**), the odor changes to that of freshly baked bread (Rosenkranz *et al.* 1963). When one of the methyl groups is missing, as in 5-methyl-4-hydroxy-3(2*H*)-furanone (**19**), the caramel-like odor is still retained, but a cooked beef-like odor is added (Tonsbeek *et al.* 1968, and Nunomura *et al.* 1979). When one of the methyl groups is replaced by an ethyl group, as in 2-methyl-5-ethyl-4-hydroxy-3(2*H*)-furanone (**20**), the caramel-like odor is still retained, but a soy sauce-like aroma is now exhibited (Nunomura *et al.* 1980). Shigematsu *et al.* (1971) have found a series of lactones which have considerably different structures but with similar caramel and soy sauce aromas. If the functional groups are changed around to form 2-acetyl-3-hydroxyfuran (**21**), or isomaltol, the odor is still described as sweet, but with a burnt sugar and fruity character (Hodge 1967). If the functional groups are rearranged to form a lac-

tone, 4,5-dimethyl-3-hydroxy-2-(5*H*)-furanone (**22**), or 2-hydroxy-3-methyl- γ -pent-2-enolactone, or sotolon, a trivial name from "soto-" (raw sugar in Japanese) and "-olon" (enol lactone), the odor is a strong raw sugar character (Tokimoto *et al.* 1980). This compound was previously found in aged sake and thought to be characteristic of burnt flavoring (Takahashi *et al.* 1976). If the methyl group in the 5-position is changed to an ethyl group, as in 2-methyl-5-ethyl-3-hydroxy-2-(5*H*)-furanone (**23**), the sugary note is diminished and a curry-like odor is detected and was reported by Sulser *et al.* (1967) as probably the flavoring principle in vegetable protein hydrolysates. The above structure-odor relationships are summarized in Table 15.1, and chemical structures are shown in Fig. 15.4.

Shigematsu *et al.* (1971) have isolated and characterized a series of very interesting pyrrole lactones (Fig. 15.5) which are the result of

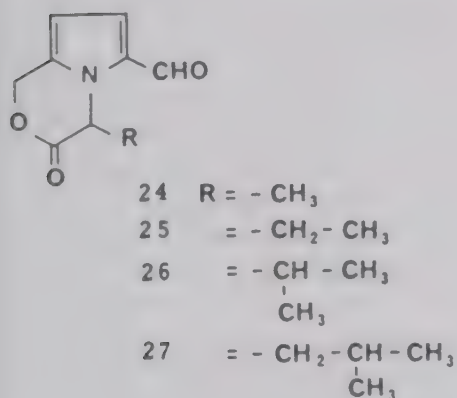


FIG. 15.5. Pyrrole lactone compounds.

roasting D-glucose and several alkyl- α -amino acids. It is difficult to relate these chemical structures to the hydroxyfuranones shown in Table 15.1, but 2-(5-hydroxymethyl-2-formylpyrrol-1-yl)-propionic acid lactone (**24**, Fig. 15.5) is reported to have a caramel and slightly scorching aroma; *n*-butyric acid lactone (**25**, Fig. 15.5) to have a maple and strong sweet aroma; and isovaleric acid lactone (**26**, Fig. 15.5) and isocaproic acid lactone (**27**, Fig. 15.5) to have a miso, soy sauce, and somewhat chocolate-like aroma. A series of these lactones have been found in raw cane sugar by Abe *et al.* (1978).

Roasted Aroma Compounds

Flavors from food materials exposed to high temperatures, such as in roasting of nuts, meats, coffee, and cocoa, are very complex and

elusive. There is no lack of effort in this area because many compounds have been isolated and identified. For a matter of convenience, the compounds of interest will be limited to two categories: cracker-like and roasted-like.

Cracker-like. A number of compounds have been identified in processed or cooked foods that have been described as possessing "cracker"-, "bread crust"-, or "popcorn"-like aromas. This is an important group of compounds because of the potent and characteristic nature of this odor. The presence of this odor has been connected with the freshness of the processed product such as with freshly baked bread (Hunter *et al.* 1969). The main chemical feature common to this group is that (with one or two exceptions) they all possess an acetyl group attached to a heterocyclic ring containing nitrogen, with the acetyl group attached to the α position compared to the nitrogen. All the compounds seem to be produced by the interactions occurring during the heating of the combination of amino acids and sugars. Probably one of the most representative members of this group is 2-acetyl-1,4,5,6-tetrahydropyridine (30, Fig. 15.6). This was first isolated as a fraction from bread crust (Hunter *et al.* 1969). The bread component was too small to characterize, and a component with the same odor and gas-liquid chromatography properties was isolated by heating the amino acid proline with dihydroxyacetone and sodium bisulfite. The compound was first synthesized in a systematic way by Buchi and Wuest (1971).

Another important member of this group, 2-acetylpyrazine (33, Fig. 15.6), was first patented as a popcorn-like aroma component of tobacco (Roberts 1968). This compound was later tentatively identified in popcorn by Waldradt *et al.* (1970). 2-Acetylpyrazine and its alkyl-substituted derivatives were also later found in a number of cooked products such as roasted coffee (Vitzthum and Werkhoff 1974), roasted almonds (Takei and Yamanishi 1974), and cooked pork liver (Mussinan and Waldradt 1974).

A third important cracker aroma-like compound was identified in the volatiles from beef broth as 2-acetyl-2-thiazoline (29, Fig. 15.6) by Tonsbeek *et al.* (1971), who considered it to have an "intense aroma of freshly baked bread crust."

Two early identified members of this group are *N*-acetylpyrrolidine and *N*-acetylpyrrole (Kobayashi and Fujimaki 1965), which were formed by heating hydroxyproline with glucose. This work was later confirmed by Tressl *et al.* (1981).

The compounds 2-acetylpyridine (32, Fig. 15.6) and 2-acetylthiazole (31, Fig. 15.6) have been found in a considerable number of cooked

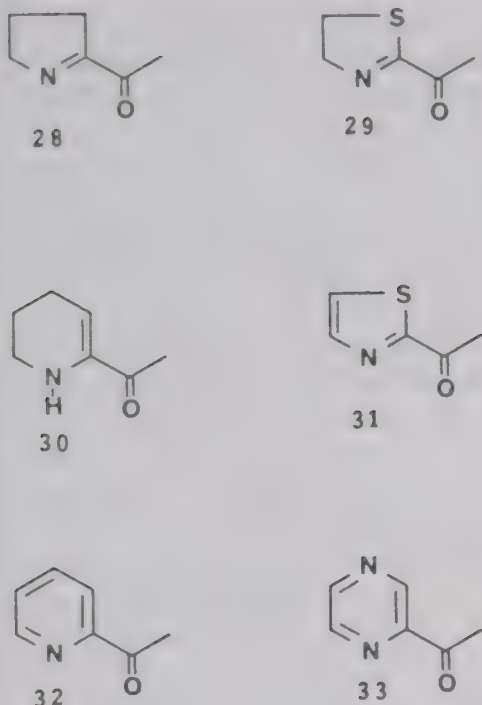


FIG. 15.6. Cracker odor compounds. Names are listed in Table 15.2.

foods (e.g., Buttery *et al.* 1975). These may be formed from 2-acetyl-1,4,5,6-tetrahydropyridine and 2-acetyl-2-thiazoline by air oxidation during the isolation of these from the food. Both possess cracker-like aromas, although they are considerably weaker and less characteristic than the partially hydrogenated forms. 2-Acetylpyrrole has been found in a considerable number of cooked foods (cf. Mussinan and Waldradt 1974); however, this compound has relatively little odor. Its partially hydrogenated form 2-acetyl-1-pyrroline (**28**, Fig. 15.6), on the other hand, is the most potent cracker odor compound yet found and has a very characteristic popcorn-like aroma. 2-Acetyl-1-pyrroline has been shown (Buttery *et al.* 1983) to be largely responsible for the aroma of the aromatic (scented) Asian varieties of rice (e.g., Basmati).

All of the cracker odor group of compounds occur only in processed or cooked foods and seem to result from some form of Maillard reaction. Hodge *et al.* (1972) have outlined a possible mechanism for the production of 2-acetyl-1,4,5,6-tetrahydropyridine, 1-pyrroline, and *N*-acetyl-2-pyrrole from the reaction of pyruvaldehyde (a sugar degradation product) with the amino acid proline. It seems reasonable that a related mechanism might lead to the production of 2-acetyl-1-pyrroline from proline or hydroxyproline.

Tressl *et al.* (1981) have thoroughly studied the volatile compounds formed on heating proline and hydroxyproline with glucose and maltose. Their results have supported the general mechanisms outlined by Hodge *et al.* (1972).

There are considerable differences between the odor potencies of the different cracker odor compounds. Table 15.2 lists the odor thresholds that have been determined for some of these compounds. These data were taken from Teranishi *et al.* (1975) and Buttery *et al.* (1983). It can be seen that the compounds with partially hydrogenated rings are much more potent odorants than those with completely aromatic rings. The availability of the "lone pair" of electrons on the nitrogen atom may be important to the cracker odor.

TABLE 15.2. ODOR THRESHOLDS OF SOME CRACKER ODOR COMPOUNDS IN WATER SOLUTION^a

Compound	Odor threshold (μ g/liter water)
(28) 2-Acetyl-1-pyrroline	0.1
(29) 2-Acetyl-2-thiazoline	1.3
(30) 2-Acetyl-1,4,5,6-tetrahydropyridine	1.6
(31) 2-Acetylthiazole	10.0
(32) 2-Acetylpyridine	19.0
(33) 2-Acetylpyrazine	62.0

^a Chemical structures of compounds are shown in Fig. 15.6 and correspond to the boldface numbers given here.

Roasted-like. As the world population increases, the need to utilize plant protein directly becomes more important because of the poor efficiency of converting plant protein to animal protein. Conversion of soybean protein by fermentation to meat-like flavored products has been discussed briefly in the soybean section. There is also a need to flavor meat-textured vegetable protein food products to make them more palatable. Some meat-like aromas have been discussed already in the nonenzymatic browning section in which Tonsbeek *et al.* (1971) have reported that 5-methyl-4-hydroxyl-3(2*H*)-furanone has a beef-like aroma, and Sulser *et al.* (1967), have reported that 2-methyl-5-ethyl-3-hydroxy-2(5*H*)-furanone is the principal flavoring agent in vegetable protein hydrolysates. These are, however, a very small part of the compounds found in roasted materials and cooked meats because various arrays of heteroatomic and heterocyclic compounds associated with the Maillard-type reactions (Wilson *et al.* 1973) must be considered.

In their usual elegant manner, Ohloff and Flament (1978A, B) and Flament (1975) reviewed the role of heteroatomic substances and het-

erocyclics in aroma compounds in foods. Vernin (1982) has edited an excellent book on heterocyclic compounds in flavors and aromas. Maga and Sizer (1973A, B) and Maga (1975A, B, 1976A, B, 1979, 1981) have compiled reviews of the role of pyrazines, thiazoles, oxazoles, etc., in foods. Pittet and Hruza (1974) made a comparative study of flavor properties of some thiazoles, and Shibamoto (1980) reviewed heterocyclic compounds found in cooked meats. Rijkens and Boelens (1975) have tabulated the research in meat flavor (Fig. 15.7), and of over 500 compounds known in 1975, about 20% were nitrogen and sulfur compounds. Dwivedi (1975) reviews and lists the large number of compounds isolated from heat-treated beef and discusses patents on synthetic meat flavors and origin of meat flavor. However, it is pointed out that what contributions the many compounds listed make to the "typical" meat flavor are uncertain. Ohloff and Flament (1978B) describe the sensory properties of many heterocyclic constituents of meat odor, but also point out that meat aroma cannot be attributed to a single compound or even a particular class of compounds.

No simple comparisons can be made in the roasted aromas as in the caramel-like or cracker-like series. However, there is no doubt that pyrazines, thiazoles, oxazoles, etc., play important roles in roasted

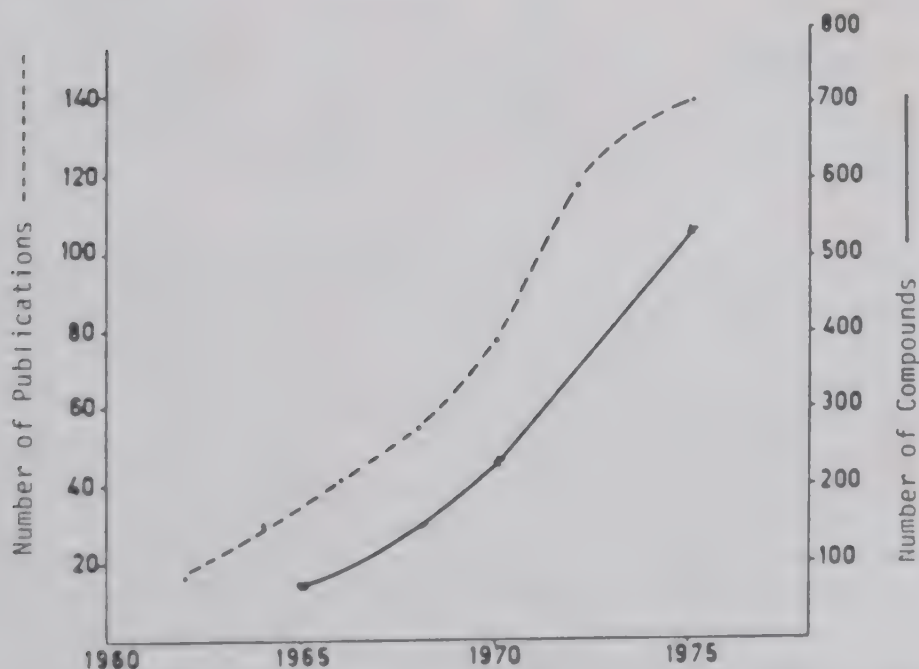


FIG. 15.7. Research on meat flavor in terms of number of publications and number of compounds.

aromas. One of the difficulties is that these compounds exhibit a range in properties, from thresholds to odor characteristics.

In Table 15.3, odor thresholds of some pyrazines are listed with some very brief descriptions of the characteristics. The range of 10^8 in thresholds indicates that some pyrazines will have much effect at very low concentrations, while some will have little effect at much higher concentrations. Therefore, it is important to know not only the characteristics of compounds, but also at what level they will contribute significantly to the overall aroma. The odor characteristics range from green bell pepper, 2-methoxy-3-isobutylpyrazine, to roasted peanuts, 2-methoxy-3-methylpyrazine, to green fruity, 2-isobutylpyrazine. It is remarkable that such small changes in structure make such large changes in character and threshold.

TABLE 15.3. ODOR THRESHOLDS AND CHARACTERISTICS OF SOME PYRAZINES

Compounds	$\mu\text{g/liter water}$	Characteristic	References
2-Methoxy-3-hexylpyrazine	0.001		Teranishi (1971)
2-Methoxy-3-isobutylpyrazine	0.002	Green bell pepper	Teranishi (1971)
2-Methoxy-3-isopropylpyrazine	0.002	Potato	Teranishi (1971)
2-Methoxy-3-propylpyrazine	0.006	Pepper	Teranishi (1971)
2-Methoxy-3-ethylpyrazine	0.4	Raw potato	Teranishi (1971)
2-Methoxy-3-methylpyrazine	4.0	Roasted peanuts	Teranishi (1971)
2-Isobutylpyrazine	400.0		Teranishi (1971)
2-Methoxypyrazine	700.0	Sweet, nutty	Teranishi (1971)
			Pittet and Hruza (1974)
2,5-Dimethylpyrazine	1,800.0		Teranishi (1971)
2,3-Dimethylpyrazine	2,500.0	Green, nutty	Pittet and Hruza (1974)
2-Ethylpyrazine	6,000.0	Nutty, roasted	Pittet and Hruza (1974)
2-Methylpyrazine	60,000.0	Nutty, roasted	Pittet and Hruza (1974)
Pyrazine	175,000.0		Teranishi (1971)

In Table 15.4, the odor thresholds and some characteristics of some thiazoles are listed. With data available, these compounds do not seem to exhibit as large a range of thresholds as do the pyrazines. However, these compounds play an important role in aromas for meat, coffee, cocoa, and so on (Ohloff and Flament 1978B).

The compounds in Tables 15.3 and 15.4 are but a small part of the series represented, and there are at least 26 different basic skeletons of heterocyclic compounds found in meat flavors (Ohloff and Flament 1978B). Even though 500–600 compounds have been identified in meat-like flavors (Rijkens and Boelens 1975, Ohloff and Flament 1978B), perhaps some key compounds are still to be discovered, compounds with thresholds lower than those found so far. Of the many compounds known, the task remains to sort out which are the most important. An enormous systematic job lies ahead.

TABLE 15.4. ODOR THRESHOLDS AND CHARACTERISTICS OF SOME THIAZOLES

Compounds	$\mu\text{g/liter water}$	Characteristics	References
4-Butyl-5-propylthiazole	0.003	Bell pepper	Buttery <i>et al.</i> (1976)
4-Butyl-5-methylthiazole	0.01	Bell pepper	Buttery <i>et al.</i> (1976)
4-Butyl-5-ethylthiazole	0.02	Bell pepper	Buttery <i>et al.</i> (1976)
4-Ethyl-5-propylthiazole	0.06	Bell pepper	Buttery <i>et al.</i> (1976)
2,5-Diethylthiazole	0.09	Skunky	Buttery <i>et al.</i> (1976)
2,4-Dimethyl-5-ethylthiazole	0.09	Nutty, roasted, meaty	Teranishi <i>et al.</i> (1974)
4-Ethyl-5-butylthiazole	0.12	Bell pepper	Dittet and Hruza (1974)
4-Pentyl-5-butylthiazole	0.19		Buttery <i>et al.</i> (1976)
4,5-Diethylthiazole	5.1		Buttery <i>et al.</i> (1976)
2,4-Diethylthiazole	6.5	Musty, earthy	Teranishi <i>et al.</i> (1975)
2-Acetylthiazole	10.0	Cracker	Buttery <i>et al.</i> (1976)
2,4-Dimethylthiazole	18.0	Skunky	Teranishi <i>et al.</i> (1975)
2,4-Dipropylthiazole	26.0	Sweet, fruity	Teranishi <i>et al.</i> (1975)
4-Methyl-5-ethylthiazole	100.0	Nutty, green, earthy	Teranishi <i>et al.</i> (1974)
4-Acetylthiazole	170.0		Pittet and Hruza (1974)
4-Propylthiazole	300.0		Teranishi <i>et al.</i> (1975)
4,5-Dimethylthiazole	470.0	Roasted, nutty, green	Teranishi <i>et al.</i> (1974)
			Buttery <i>et al.</i> (1976)
			Pittet and Hruza (1974)
Thiazole	3100.0		Teranishi <i>et al.</i> (1974)

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Changes in Pectin and Cellulose during Processing

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INTRODUCTION

Polysaccharides provide much of the structure which gives a wide variety of foods desirable textural properties. Cellulose, pectin, and hemicellulose are the major polysaccharide components in the cell wall of all plant foods. Due to the complexities of these polysaccharides, the even greater complexity of the plant cell wall, and numerous experimental difficulties in working with these polysaccharides, much remains to be learned in establishing detailed structure-function relationships both for biological and technological functions. However, there has been rapid progress in understanding the physical and chemical properties of polysaccharides in recent years. As new information and new techniques are applied more intensively to problems of interest to food technologists, we are likely to see considerable progress in our understanding of the chemistry of food texture. The purpose of this chapter is to review recent developments in the chemistry of plant cell wall polysaccharides, particularly pectin and cellulose, as they may relate to the texture of fruits and vegetables.

CELL WALL STRUCTURE

There have been major advances in our understanding of plant cell wall structure during the past 15 years. This progress has been made

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possible by improvements in the methods available to analyze complex polysaccharides. For example, Waeghe *et al.* (1983) have recently published procedures which require only 5 μ g of starting material to analyze sugar composition and linkage patterns in cell wall polysaccharides by using capillary gas chromatography and mass spectroscopy. Advances are also being made in mass spectrometry of underivatized carbohydrates (Dell *et al.* 1983) and structural analysis of complex carbohydrates using both ^{13}C nuclear magnetic resonance (NMR) (Barker *et al.* 1982) and proton NMR (Dabrowski *et al.* 1982), so that it is now possible to do sequence analysis of complex polysaccharides (McNeil *et al.* 1982). Darvill *et al.* (1980) and McNeil *et al.* (1984) have presented detailed reviews of studies of cell wall structure, particularly of the work in Albersheim's laboratory. Table 16.1 shows the major components in suspension-cultured sycamore cell walls.

TABLE 16.1. POLYMER COMPOSITION OF THE WALLS OF SUSPENSION-CULTURED SYCAMORE CELLS

Wall component	Weight (%) of cell wall
A. Pectic polysaccharides	34
Rhamnogalacturonan I	7
Homogalacturonan	6
Arabinan	9
Galactan and possible arabinogalactan	9
Rhamnogalacturonan II	3
B. Hemicelluloses	24
Xyloglucan	19
Glucuronoarabinoxylan	5
C. Cellulose	23
D. Hydroxyproline-rich glycoprotein	19

From Darvill *et al.* (1980).

Cellulose has a partially crystalline, fibrous structure to provide the basic structural strength of the cell wall. Pectic polysaccharides and hemicelluloses constitute an amorphous matrix around the cellulose fibers. Specific structural roles for the individual fractions of these matrix components remain to be defined. Lamport (1965) originally suggested that hydroxyproline-rich glycoproteins control the extension of cell walls during plant growth and suggested the name "extensin" for these glycoproteins. However, their functional role also remains to be defined (Lamport 1980).

All plant cell walls contain the four major components listed in Table 16.1. Monocot plants differ from dicots principally because they have a much lower content of pectic substances and a higher content of xylans. From the viewpoint of trying to define basic principles of cell wall structure and function, Darvill *et al.* (1980) have emphasized the similarities of cell wall polysaccharides among higher plants.

However, if we are to explain the wide variety of textures in fruit, vegetable, and cereal products, I suspect food technologists will also have to appreciate the considerable dissimilarities in cell wall composition which appear to occur. Table 16.2 is a compilation of the sugar and uronic acid composition of a variety of fruit and vegetable cell walls which have recently been analyzed by Voragen *et al.* (1983) and Selvendran and co-workers (Selvendran 1983). Within this group of products, there are large variations in the relative amounts of each sugar present in the cell wall, with a range of 2.8-fold for glucose to 17.7-fold for xylose. This is an indication of the magnitude of variability in cell wall components that occur in foods. There may also be substantial variability in the size, branching, and sugar composition within the polysaccharide structure. The pectic substances will vary in degree of carboxyl methylation. In addition, there will be differences in the amount and types of proteins and lignin present.

It is not surprising, in view of the many possibilities for variation in cell wall structure, that there is much to be learned about structure-function relationships. A recent development which may add another useful tool to the techniques for understanding polysaccharide changes has been the use of 4-methylmorpholine *N*-oxide (MMNO) to dissolve cellulose and cell walls. Joseleau *et al.* (1981) showed that cellulose dissolved in MMNO underwent little or no degradation, as indicated by methylation analysis of the dissolved polymer. They also found that primary cell walls from suspension-cultured plant cells could be almost completely dissolved in MMNO and then remain in solution when diluted with dimethyl sulfoxide. This group has recently utilized MMNO to partially fractionate cell wall components (Chambat *et al.* 1984). The ability to dissolve these polymers without degradation offers the opportunity to fractionate complex mixtures of polysaccharides in ways that were previously impossible.

By far, the greatest amount of research on the role of cell wall components in food texture has been done on pectic substances. As a result, pectin chemistry will be the major focus of this review. Cellulose has received little attention. Hemicellulose polysaccharides and cell wall structural proteins have not been available in sufficient quantity to even seriously begin investigations into their role in texture and texture changes in fruits and vegetables.

SUGAR COMPOSITION, SIZE, AND SHAPE OF PECTIN MOLECULES

Pectin molecules have a backbone of linear chains of galacturonic acid residues in α -1,4-glycosidic linkages. Rees and Wight (1971) de-

TABLE 16.2. SUGAR COMPOSITION OF CELL WALLS FROM VARIOUS FRUITS AND VEGETABLES^a

	Reference ^b	6-Deoxyhexose ^c	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acid	Total ^d
Potatoes	1	20.4	46.1	16.5	8.3	284	315	270	960
Runner beans	1	19.3	35.1	17.3	15.7	74.8	314	367	843
Apples	1	19.6	122.8	33.3	43.0	56.8	227	328	830
Apples	2	18	103	58	20	67	251	239	756
Raspberries	2	6	23	45	15	22	158	148	417
Strawberries	2	9	47	35	19	44	147	274	575
Cherries	2	8	91	12	11	36	124	308	590
Papaya	2	8	12	22	26	48	268	357	741
Mango	2	9	41	55	13	36	236	322	712
Pear	2	10	42	129	16	31	206	171	605
Carrots	2	11	67	9	16	78	259	312	752
Cucumber	2	7	22	36	20	62	291	216	654
Onions	2	9	26	13	—	201 ^e	210	229	688
Pineapple	2	2	101	159	20	65	351	66	764
Ratio (high/low)		10.2	10.2	17.7	5.2	12.9	2.8	5.6	

^a Neutral sugars analyzed after Saeman hydrolysis. Uronic acid measured colorimetrically. Results expressed as $\mu\text{g}/\text{mg}$ of isolated cell wall.^b 1, Selvendran (1983); 2, Voragen *et al.* (1983).^c Combination of rhamnose and fucose.^d The totals do not include protein and lignin which often are present in substantial quantities in cell wall preparations. See Voragen *et al.* (1983).^e Combination of mannose and galactose.

defined the major conformational characteristics of polygalacturonate chains. The uronic acid residues have a helical arrangement with exactly three monomers per turn of the helix. The glycosidic oxygen atom is in an axial position relative to the plane of the monomer. This has the effect of forming a buckled ribbon conformation (Rees 1977) which is quite rigid because any rotation about the glycosidic bonds will cause contact between groups on adjacent residues. The somewhat buckled shape of the molecule is indicated by the fact that the residue length projected on the helix axis is only 4.35 Å compared to a residue length of 5.15 Å for glucose residues in cellulose, which has a more extended ribbon conformation (Gardner and Blackwell 1974).

A general feature of all gel-forming polysaccharides is that they have structural irregularities which reduce the regularity of inter-chain associations (Powell *et al.* 1982). If this did not occur, polysaccharides such as pectin, alginate, or carageenan would probably form condensed, insoluble precipitates rather than hydrated gels with variable firmness and rigidity. Irregularities in the structure of pectin are caused by variable methylation of the carboxyl groups of galacturonic acid residues, neutral sugar side chains, and occasional rhamnose residues in the main chain of the molecule. Using conformational calculations, Rees and Wight (1971) found that rhamnose causes a pronounced kink in a chain of galacturonic acid residues (Fig. 16.1). It is still not known whether rhamnose in pectin has an α - or β -configuration, but both configurations result in formation of a kink. The distance between rhamnose kinks is not known with certainty. Indeed, it could be an important factor in pectin variability among species. However, Powell *et al.* (1982) prepared block polymers from citrus, apple, and sunflower pectin by hydrolysis in 0.25 M H₂SO₄ at 100°C for 3 hr. Based upon the relative instability of rhamnosyl glycosidic linkages compared to galacturonic acid bonds, they suggested that hydrolysis occurred primarily at rhamnose residues. Since the hydrolyzed oligomers had a narrow size range and were about 25 residues long, it suggests that, in these pectins at least, rhamnose residues are located about 25 galacturonic acid residues apart.

Except for highly degraded samples, neutral sugars have always been found in pectin. It is likely that commercial pectin preparations, which have been used in almost all physicochemical studies on pectin, will generally contain less neutral sugars than native pectin because hot acid is normally used for commercial extraction. Table 16.3 shows the data of Voragen *et al.* (1983) for the neutral sugars in pectin extracted from the alcohol-insoluble solids of fruits and vegetables by 5 mM EDTA in cold 50 mM NaOH. Included for comparison are the data of Fishman *et al.* (1984A) for a dialyzed sample of commercially

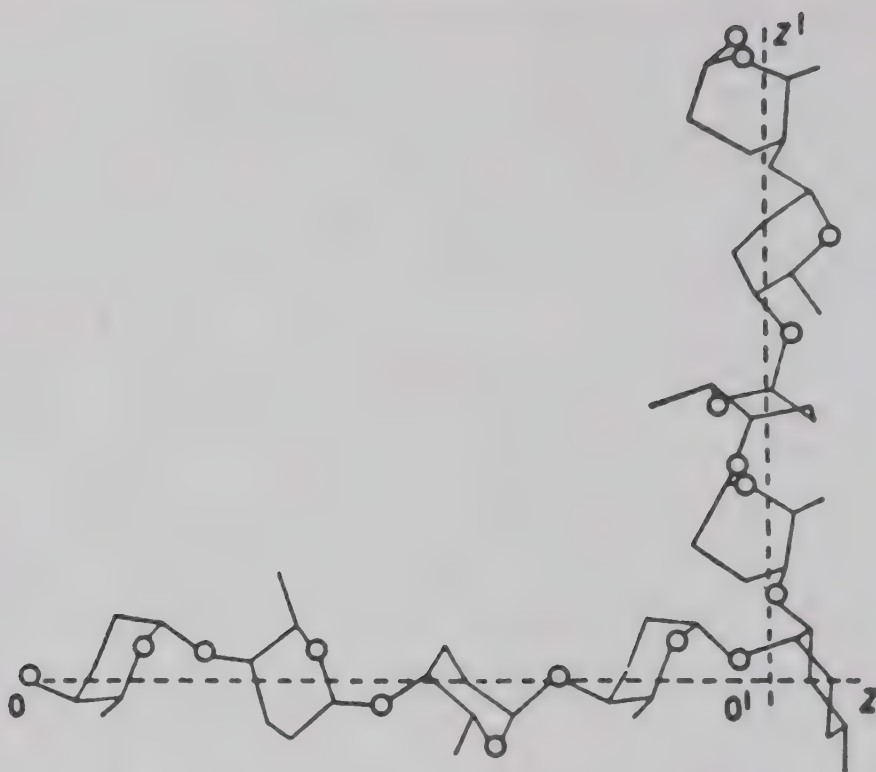


FIG. 16.1. Effect of inserting a single β -L-rhamnose unit, linked through its 2 position, into a galacturonan chain. The rhamnose residue is located at the point where the chain kinks.

From Rees and Wight (1971).

prepared pectin. The most notable difference between the commercial sample and the other pectin extracts is the higher proportion of rhamnose, suggesting a larger proportion of kinks in the uronic acid chains. Just as was the case for total cell wall sugars, the relative proportions of sugars from the different tissues varied over a severalfold range. Studies on the effects of neutral sugar composition upon the physical properties of pectin remain to be done.

The molecular weight of pectin preparations has been reported to vary from 6100 for a commercial sample of citrus polypectate (Fishman *et al.* 1984A) to 366,000 for pectin isolated from unripe peaches (Shewfelt *et al.* 1971). Estimates of pectin molecular weights have been limited by two serious problems. The first is that pectin is normally tightly held in the cell wall matrix. Therefore, it is likely that any extracted pectin which can be used for molecular weight determinations is partially degraded. However, there is no way at present to assess the extent of any degradation which occurs during extrac-

TABLE 16.3. SUGAR COMPOSITION (MOL %) OF A COMMERCIAL PECTIN SAMPLE AND THE PECTIN FRACTIONS ISOLATED FROM CELL WALLS OF FRUITS AND VEGETABLES

Commodity	6-Deoxyhexose ^a	Arabinose	Xylose	Mannose	Galactose	Glucose	Uronic acid
Commercial pectin ^b	7.00	2.03	1.12	1.32	8.49	2.03	78.01
Raspberries ^c	1.6	3.4	2.6	5.5	3.5	4.9	78.5
Strawberries	2.3	9.5	1.5	—	8.4	6.9	71.4
Cherries	2.3	20.6	1.4	2.1	5.9	1.5	66.3
Papaya	3	4.5	4.5	1	6.7	1	79.3
Pineapple	0.7	29.8	36.5	0.6	9.7	4.2	18.4
Mango	1	15.5	1.8	0.6	4.4	2.7	74
Apple	1.4	23.3	2	0.4	4.1	1.3	67.5
Pear	1.8	21.5	2.5	0.8	3.2	11	59.2
Carrots	1	7.9	—	—	11.8	2	77.4
Cucumber	0.6	5.8	2.2	—	11	1.8	78.7
Onions	0.6	3.8	2.3	0.6	40	4.4	48.3

^a Combined rhamnose and fucose.

^b From Fishman *et al.* (1984A). The 6-deoxyhexose is only rhamnose.

^c All fruit and vegetable data from Voragen *et al.* (1983).

tion. The second problem is that pectin aggregates in aqueous solution (Sorochoan *et al.* 1971; Fishman *et al.* 1984A). The extent of aggregation can be affected by the extent of methylation, presence of neutral sugars, pH, and ionic strength of the medium. Aggregation will result in overestimating molecular weights using viscometry, ultracentrifugation, light scattering, or osmometry.

Fishman *et al.* (1984A) determined the number average molecular weight for several pectin samples by using ^{13}C NMR spectroscopy to measure the galacturonic acid content and a reducing end group assay to determine the number of pectin molecules. Neither of these determinations should be affected by the aggregation of pectin. Molecular weights ranging from 6100 to 15,200 were obtained. The accuracy of this procedure would be expected to decrease as molecular weights increase because the proportion of reducing groups in a sample will become very small. Fishman *et al.* (1984B) found a molecular weight of 6900 using osmometry in 0.05 M NaCl for the same polypectate sample for which a 6100 molecular weight had been obtained by chemical determination. This indicated that 0.05 M NaCl prevented aggregation of this sample. Unfortunately, there is not a single solvent system which will assure the disaggregation of pectin samples for physical determinations of molecular size.

PECTIN METHYLATION

Table 16.4 indicates that the extent of methyl esterification of pectin can vary over a wide range in fruit and vegetable tissues. It also indicates that two procedures which are used to measure esterification can give quite different results. However, regardless of the analytical procedure employed, in no case has completely esterified or deesterified pectin been isolated from cell walls. Few details of the mechanism of methylation *in vivo* are known. At one time, it was believed that galacturonic acid methyl ester was incorporated into the galacturonan chain during synthesis (Albersheim and Bonner 1959), but more recent evidence appears to favor methylation after polymer formation (Kauss and Hassid 1967). *S*-Adenosylmethionine is the source of the methyl groups in pectin (Kauss *et al.* 1969). It is not known whether, as a general rule, galacturonic acid residues are methylated randomly or by some defined pattern. However, recent analysis of the statistical distribution of methyl groups in apple pectin indicates a random distribution of esterified carboxyl groups (DeVries *et al.* 1983B). Despite the high level of pectinesterase present in the albedo of citrus fruits, a random distribution of methyl

groups was also found in pectin from lemon (DeVries *et al.* 1984). Since pectinesterases from higher plants do a sequential hydrolysis of methyl groups from pectin to give blocks of demethylated residues (Rexova-Benkova and Markovic 1976), these results suggest that galacturonic acid residues may be methylated by a random mechanism.

Chemical procedures have been developed for both esterification and deesterification of isolated pectin. The most common methylation techniques are esterification in acidified methanol (Heri *et al.* 1961) or use of diazomethane (Pfeffer *et al.* 1981). There is recent evidence that diazomethane can also form methyl esters on the secondary hy-

TABLE 16.4. DEGREE OF ESTERIFICATION OF URONIDES ESTIMATED FROM METHANOL RELEASE AND COPPER BINDING BY THE PECTIN FRACTION

	Methanol release	Copper binding
Raspberries	20 ^a	55
Strawberries	60	88
Cherries	36	44
Papaya	56	68
Pineapple	22	25
Mango	78	79
Apple	72	81
Pear	51	61
Carrots	45	63
Cucumber	51	73
Onions	50	78

From Voragen *et al.* (1983).

^a Percentage esterification.

droxyl groups of galacturonic acid residues in pectin (Fishman *et al.* 1984A). Deesterification is done either by base hydrolysis at 0°C (Powell *et al.* 1982) or acid hydrolysis at room temperature (Speiser *et al.* 1945). Base hydrolysis is much more rapid, but chain scission may occur unless the temperature is carefully controlled. All of the chemical techniques result in a random distribution of methyl groups. The only selectivity that has been observed is that when a partial alkaline deesterification of pectin is carried out by reducing the temperature and limiting the reaction time, the free carboxyl groups released will be located between methyl groups whenever possible. The consequence of this pattern is that at 50% esterification of the carboxyl groups, there will be alternating free and methylated carboxyl groups (Deuel *et al.* 1953; Katchalsky and Feitelson 1954).

PECTIN IONIZATION AND ION BINDING

Many of the properties of pectins in plant cell walls and in processed foods are greatly affected by the ionization of free carboxyl groups and by the binding of metal ions. The experimental pK_a values of the free carboxyl groups of pectic acid ($\approx 0.1\%$ solution) vary from 3.6 to 4.1 as the pectic acid is titrated from the fully protonated form to the completely deprotonated form (Fig. 16.2; Cesaro *et al.* 1982). The apparent pK_a of D-galacturonic acid is 3.52. Thus, in low-acid foods the carboxyl groups of pectin will be completely ionized. However, in acid foods such as fruits or pickled vegetables, a large fraction of the carboxyl groups will be protonated and unavailable for ionic cross-linking by cations.

The ability of pectin to interact with metal ions, particularly calcium ions, is a major factor in its behavior in processed foods. Kohn

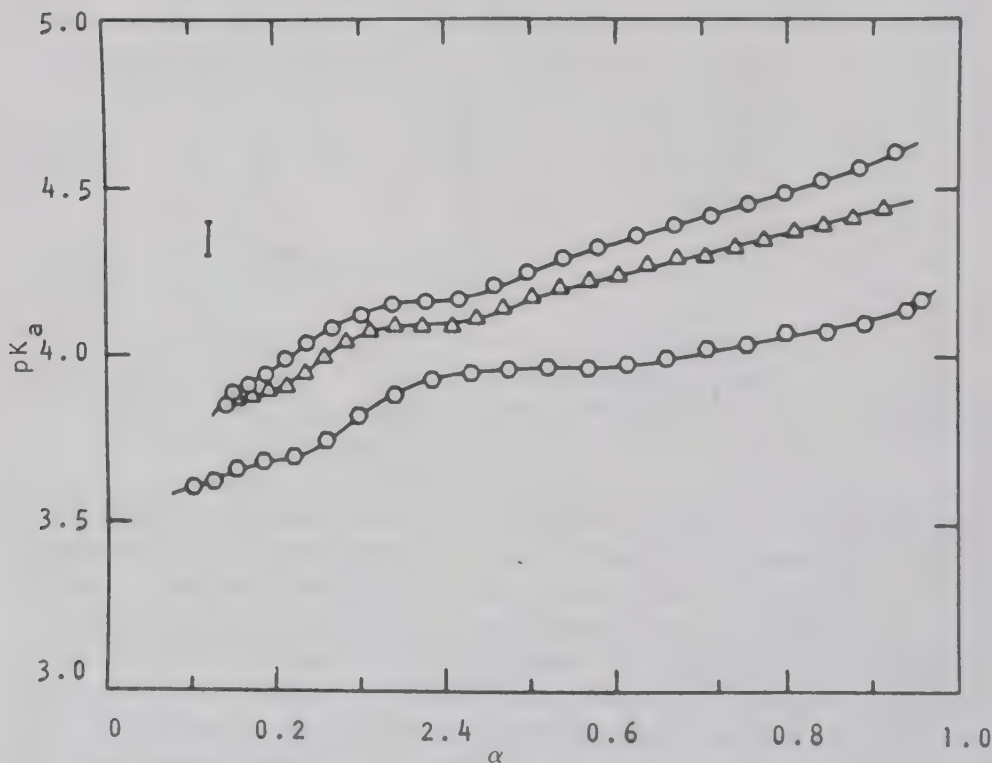


FIG. 16.2. Dependence of the apparent pK_a on the fraction of ionized carboxyl groups (α) for an aqueous solution of pectic acid titrated with 5.0×10^{-3} ($\circ$); 6.3×10^{-3} ($\triangle$); 2.13×10^{-2} ($\diamond$) equivalent /liter, respectively. The experimental pK_a value of D-galacturonic acid is 3.52. The vertical bar represents the estimated error on the absolute value of pK_a .

From Cesaro *et al.* (1982).

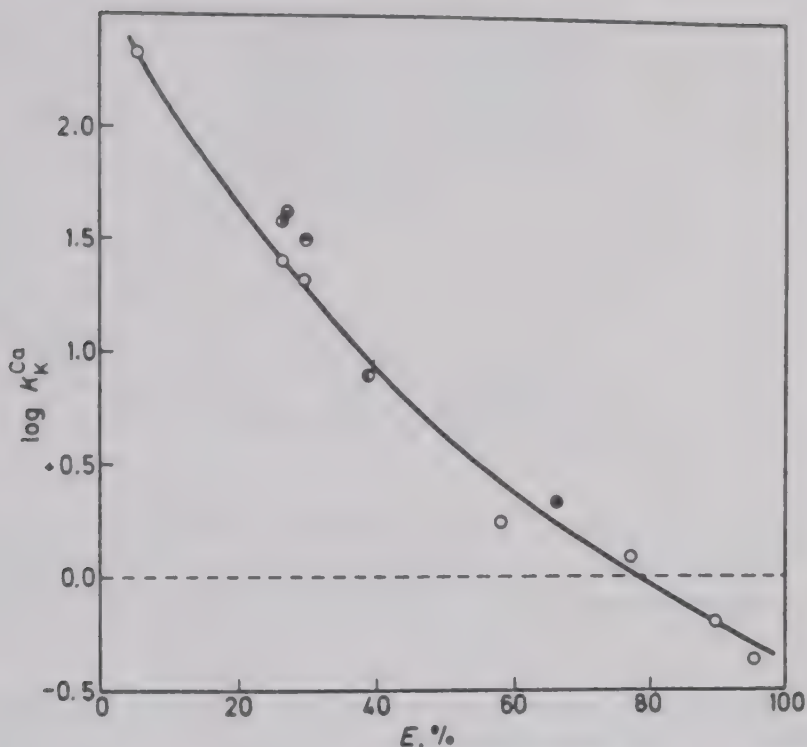


FIG. 16.3. Dependence of selectivity coefficient (K_K^{Ca}) of $Ca^{2+}-K^{+}$ exchange reaction in pectin on its degree of esterification (E).
From Kohn (1975).

and co-workers have done extensive studies of calcium binding to the potassium salt of polygalacturonans (Kohn 1975). Their major findings are that the strength of calcium binding increases as the degree of esterification decreases. Kohn (1975) defined a selectivity coefficient (K_K^{Ca}) to express the relative strength of binding of calcium ions to potassium ions in ionic polysaccharides. Figure 16.3 shows that calcium binding is not favored over potassium ion binding when the degree of esterification is about 80%, but with less than 10% esterification, the selectivity of calcium is favored by a factor of over 100. Kohn *et al.* (1975) have determined selectivity coefficients for several metal ions in cross-linked pectate. Table 16.5 shows that Pb^{2+} and Cu^{2+} bind much more strongly than calcium. The effect of oligomer size on calcium ion activity using mannuronate, guluronate, and galacturonate oligomers has also been studied (Fig. 16.4). The calcium activity in mannuronate solutions decreases as oligomer size increases as a smooth function. However, a discontinuity in the decrease of the calcium ion activity occurs with both guluronate and

TABLE 16.5. SELECTIVITY COEFFICIENTS (K_K^{Me}) FOR THE EXCHANGE OF K^+ IONS WITH DIVALENT METALS (Me) IN CROSS-LINKED POLYPECTATE^a

Metal	Selectivity coefficient
Mg	26
Ca	121
Sr	120
Co	241
Pb	2580
Cu	3300

From Kohn *et al.* (1976).

^a $\mu = 0.15$ and the mole fraction of metal ion = 0.5.

galacturonate. The point at which the discontinuity occurs has been interpreted by Grant *et al.* (1973) as the point at which cooperative binding of calcium ions into an "egg box" structure begins to occur (Fig. 16.5). This corresponds to a degree of polymerization (DP) of 14 for galacturonate and about 20 for guluronate chains.

Investigations of changes in the circular dichroism (CD) spectrum when calcium displaces sodium ions in polypectate indicate that, in the presence of excess monovalent ion (0.5 *M* monovalent ion/6 mM calcium ion), polypectate molecules form dimers in an egg box conformation, with 50% of the carboxyl groups neutralized by calcium ion (Morris *et al.* 1982). However, with more than 40% random esterification of carboxyl groups, dimerization of pectin chains does not occur. If monovalent ions are not present, the dimers will further aggregate into a gel network without changes in conformation so that the carboxyl groups will be fully occupied by calcium ions.

Powell *et al.* (1982) demonstrated the importance of calcium cross-links in calcium pectate gels by showing that the strength of the gels decreased as short blocks of polygalacturonate residues (DP $\approx$ 25) were mixed with the larger residues. It was concluded that the deesterified blocks formed cross-links with the larger polypectate molecules, but because of their small size, they could not form a strong gel network. If the DP $\approx$ 25 blocks were highly esterified (84%), gel strength was not affected. Esterified blocks would not cross-link with the large polypectate chains and, as a result, had little effect on the gel structure.

One aspect of ion binding to pectin that has not received sufficient attention is the binding of monovalent ions. It is commonly observed that polypectate solutions will gel when sodium chloride is added (McFeeters *et al.* 1980), although higher concentrations of sodium ions than calcium ions are required. With more than 50% methylation,

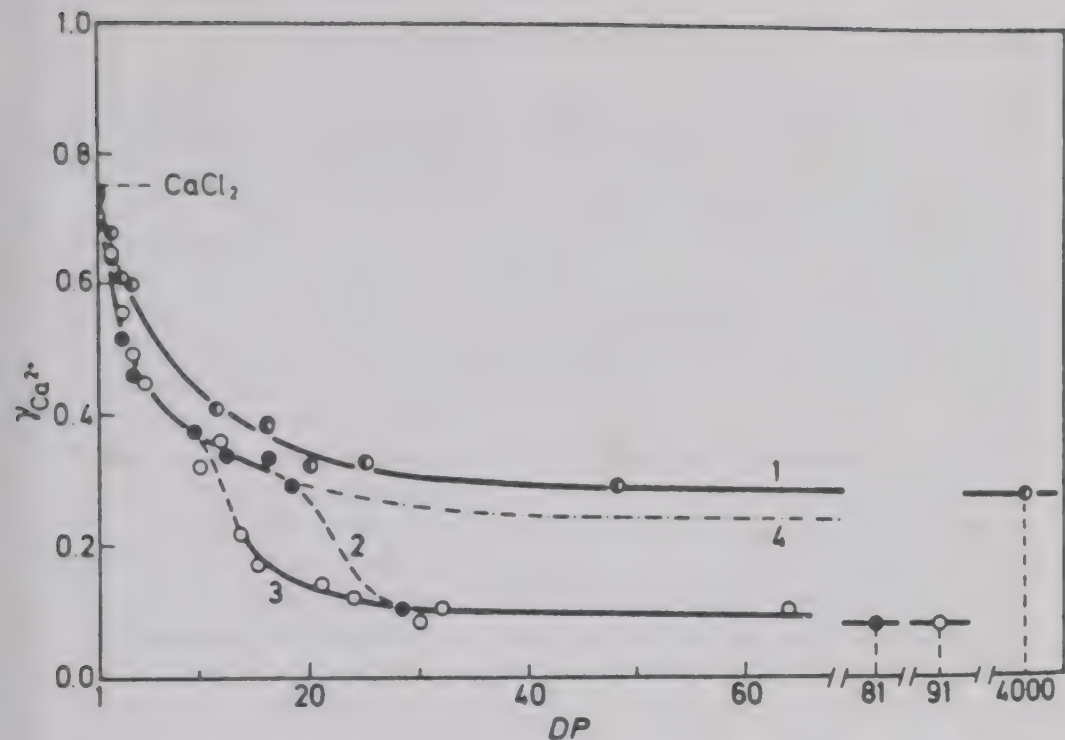


FIG. 16.4. Activity coefficient ($\gamma_{Ca^{2+}}$) in solutions of calcium oligo- and polyuronates as a function of the degree of polymerization (DP): 1, mannuronate; 2, guluronate; 3, galacturonate; 4, theoretical ($\gamma_{Ca^{2+}}$) values in solutions of calcium polyguluronate and calcium polygalacturonate.

From Kohn (1975).

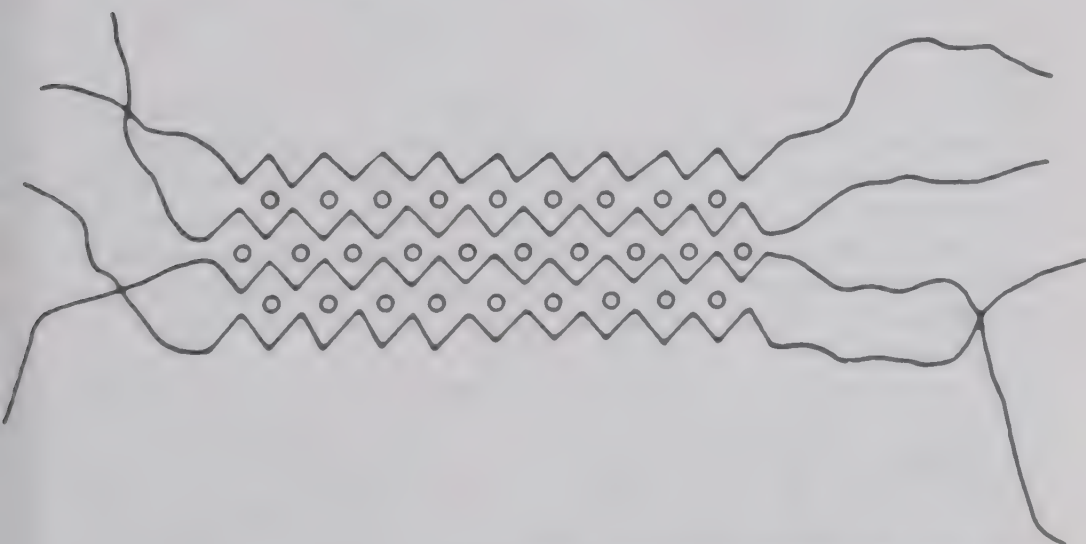


FIG. 16.5. Egg box structure formed by calcium cross-linking of polypectate chains.

From Grant et al. (1973)

pectin will not gel even in very high salt concentrations. The gelation model which has been developed for calcium and other divalent cations does not explain gelation by monovalent ions, since there is not the possibility for divalent cross-linkages.

Seale *et al.* (1982) have measured CD spectral changes and viscosity changes in alginate solutions containing various monovalent cations. Sodium ions were unique because changes in the CD spectrum were similar to those that occurred when egg box binding occurred with calcium. Furthermore, at high alginate concentrations, higher sodium ion concentrations (0.5 *M*) gave higher viscosity solutions than low sodium concentrations (0.05 *M*). In the case of potassium ion, which did not show an egg box spectrum, there was no difference in the viscosity with low and high potassium concentrations. The results were interpreted to indicate cooperative egg box binding of Na^+ between poly-L-guluronate chain sequences, which were analogous to calcium ions but considerably weaker. A model is still needed to explain the way in which a monovalent ion can cross-link polymer chains.

PECTIN STRUCTURE IN CELL WALLS

Detailed structures have not been determined for the pectic polysaccharides of any plant. The greatest amount of structural information has been obtained on pectic substances from suspension-cultured sycamore cells and apples. Recently, Stevens and Selvendran (1984) have also done an extensive analysis of pectic substances from cabbage.

Table 16.1 shows that the polysaccharide fractions, which contain a large proportion of galacturonic acid from primary cell walls of sycamore, are rhamnogalacturonan I, homogalacturonan, and rhamnogalacturonan II. McNeil *et al.* (1984) have reviewed recent work on the structures of these complex polysaccharides. Rhamnogalacturonan I contains galacturonic acid, rhamnose, galactose, arabinose, and occasionally fucose residues and has a high molecular weight with an estimated DP of about 2000 (McNeil *et al.* 1984). The main polymer chain contains alternating 1,4-linked galacturonic acid and rhamnose 1,2-linked residues. About half of the rhamnose units are branched at the 4 position with several different side chains, which average about seven sugar residues in length. The presence of homogalacturonan in the cell wall is inferred from the fact that upon treatment with an endopolygalacturonase, mono-, di-, and trigalacturonic acids are released. The sizes of the homogalacturonan chains are not

known, but partial acid hydrolysis of sycamore cell walls has resulted in the release of polymers with 15 or more residues.

Rhamnogalacturonan II is also released from cell walls by endopolygalacturonase treatment. The isolated polysaccharide contains only about 60 residues, but it is the most complex polysaccharide characterized from the cell walls. Galacturonic acid and rhamnose are the most abundant sugars, but it also contains galactose, arabinose, apiose, 2-*O*-methylfucose, glucuronic acid, 2-*O*-methylxylose, fucose, and aceric acid, the first branched-chain acidic sugar ever found in a polysaccharide.

Pectic substances from apple cell walls have been the most extensively studied pectins from fruits and vegetables. Knee (1973) and Knee *et al.* (1975) extracted a methylated polygalacturonate fraction and a branched, methylated polygalacturonate fraction containing side chains of arabinose and galactose from apple cell walls. DeVries *et al.* (1981) devised an extraction procedure which resulted in removal of 35 and 46% of the galacturonic acid from the alcohol-insoluble solids of unripe and ripe apples, respectively. The extracted pectins were subjected to extensive fractionation by DEAE-cellulose chromatography, gel filtration, and degradation by purified enzymes (DeVries *et al.* 1981, 1982, 1983A). The main conclusion of this work is that apple pectins consist of "hairy" regions with a high degree of neutral sugar branching and "smooth" regions, which are homogalacturonan sections with little or no branching. The largest portion (>90%) of the extracted pectin appears to be homogalacturonan. The hairy sections contain arabinogalactan side chains with a DP of about 25 and xylogalactan side chains with xylose attached to the galacturonan main chain. Esterification of the hairy regions of apple pectin is almost 100%, while the homogalacturonan regions have an average esterification near 70% (DeVries *et al.* 1983B). The pectic substances isolated from apple juice were found to have an average molecular weight of 95,000 with 92% of the galacturonic acid in the homogalacturonan areas and the remainder in the hairy regions (Rouau and Thibault 1984).

RECENT STUDIES OF PECTIC ENZYMES

There are several groups of enzymes produced by higher plants, molds, yeasts, and bacteria which specifically attack pectins. These include pectinesterases, which hydrolyze methyl ester groups, polygalacturonases, which hydrolyze the glycosidic bonds between galacturonic acid residues, and pectin or pectate lyases, which split poly-

galacturonan chains by a β -elimination reaction that leaves a double bond in the 4,5 position of the nonreducing terminal residue. More detailed discussions of the classification and properties of these enzymes have been published (Pilnik and Rombouts 1978; Rexova-Benkova and Markovic 1976).

The effects of pectic enzymes in food products may be desirable or detrimental, depending upon what is to be accomplished. Here, I shall briefly mention recent developments which may be important as we try to better understand and control the action of these enzymes in foods. Most interesting from a processing point of view has been the recognition that polygalacturonases, which hydrolyze the main chain of pectin, commonly are least heat stable at 50°–70°C and more stable at higher temperatures. Archer and Fielding (1975) first described such behavior for polygalacturonase from *Sclerotinia fructigena* (Fig. 16.6). Recent studies have demonstrated similar high-temperature stability for polygalacturonases from several species of *Rhizopus* and

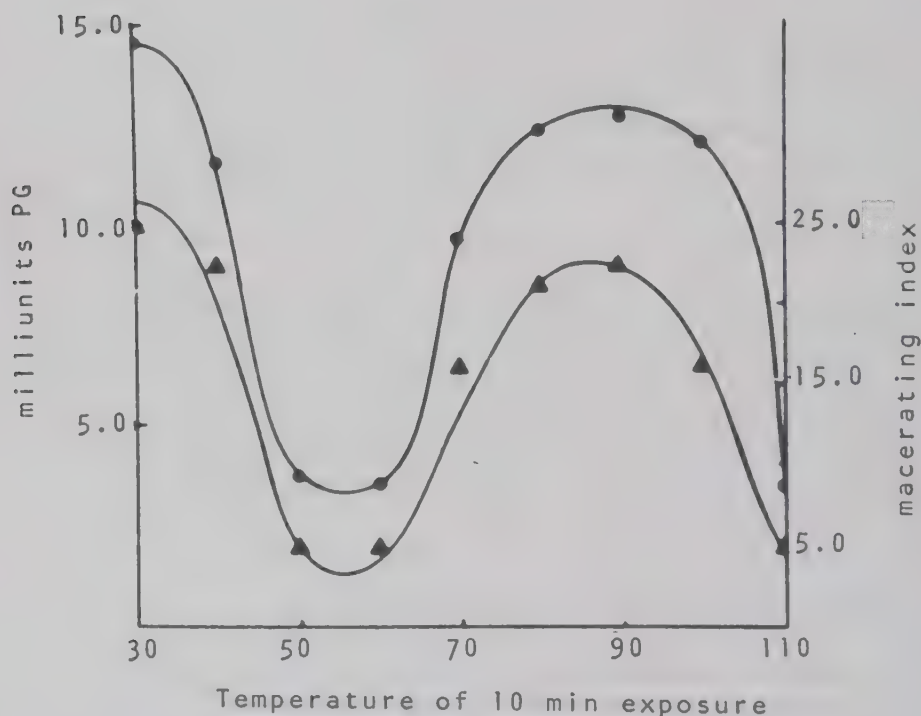


FIG. 16.6. Temperature-inactivation pattern for polygalacturonase isoenzyme from *Sclerotinia fructigena*. Residual PG activity, ●; residual macerating activity (cucumber), ▲.

From Archer and Fielding (1975).

Mucor (Harris and Dennis 1982), suggesting that bimodal heat stability may be common for fungal polygalacturonases.

It has been known for a number of years that plant pectinesterases remove methyl groups from pectin in blocks rather than by random attack, as occurs with acid or base hydrolysis of methyl esters (Rexova-Benkova and Markovic, 1976). However, Ishii *et al.* (1979) and Baron *et al.* (1980) suggested that pectinesterases from *Aspergillus japonicus* and *Aspergillus niger* removed methyl groups from pectin in a random manner. Kohn *et al.* (1983) have confirmed a random attack mechanism by a pectinesterase from *Aspergillus foetidus*. They demonstrated that pectin partially deesterified by this enzyme exhibits calcium binding properties that are nearly the same as alkali-deesterified pectin and very different from the binding characteristics of pectin treated with tomato or alfalfa pectinesterase.

The stability properties of pectinesterases from oranges and cucumbers have been the subject of recent investigations. Three forms of pectinesterase with very different stability properties have been isolated from navel oranges (Versteeg *et al.* 1980). A high-molecular-weight form of pectinesterase (MW 54,000) constituting only about 5% of the total enzyme activity in the orange requires temperatures of 90°C or higher for rapid inactivation. If it is not inactivated, it causes cloud loss in orange juice. Therefore, pasteurization processes must be designed to assure inactivation of the high-molecular-weight form of pectinesterase. The first case of reactivation of a pectinesterase has been found in cucumber slices (McFeeters *et al.* 1985). When fresh cucumber slices were blanched for 3 min at 81°C, enzyme activity could not be detected. However, when the blanched slices were stored in a pH 3.7 brine containing 0.6% acetic acid, 2.5% NaCl, and 200 ppm SO₂, about 20% of the activity present in the fresh tissue was regained during the first month of storage.

ROLE OF PECTIN IN FRUIT AND VEGETABLE TEXTURE

The importance of pectin to the strength and firmness of fruit and vegetable tissues is indicated by the fact that if tissues are treated with various polysaccharide-degrading enzymes, only polygalacturonases are capable, by themselves, of causing extensive softening and cell separation (Mussell and Morre 1969; Wallner and Bloom 1977). Work has been done to use endo-splitting polygalacturonases to produce enzymatically disintegrated vegetables for use in baby foods and vegetable juice drinks (Zetelaki-Horvath and Gatai 1977). Though there

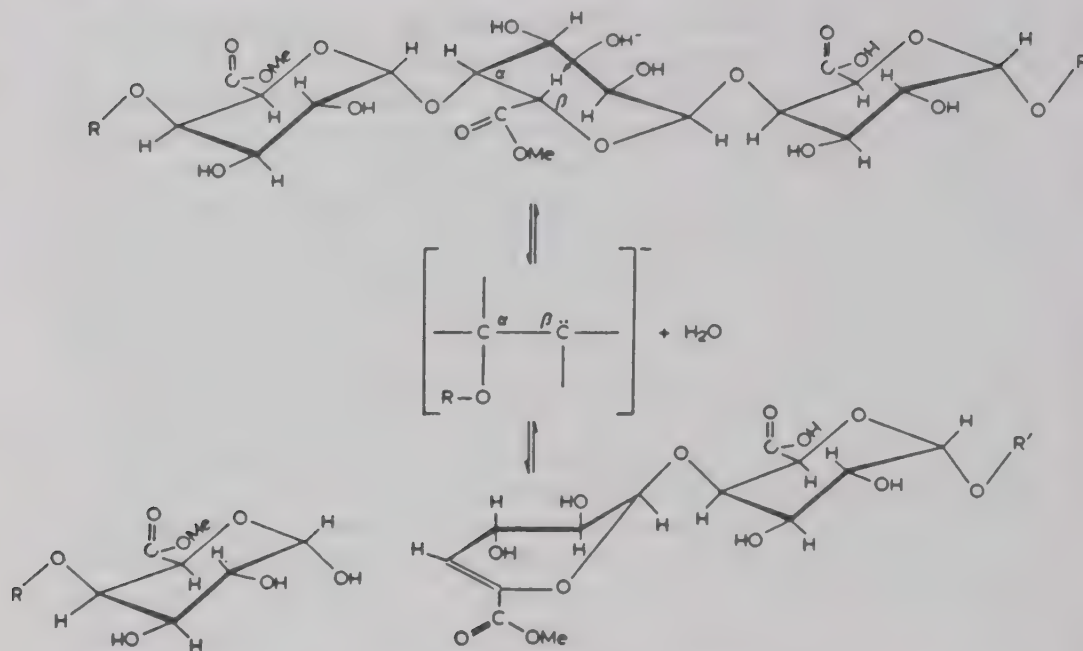


FIG. 16.7. Base-catalyzed β -elimination of esterified galacturonic acid residues. From Keijbets and Pilnik (1974).

is no doubt that pectic substances are very important to the texture of fruits and vegetables, determination of the specific ways in which changes in pectins affect texture modifications in foods is quite difficult. Plant cell walls contain a variety of polysaccharides as well as some proteins, so there are almost always problems in assigning relationships between textural changes and specific structural changes.

Albersheim *et al.* (1960) found that pectin can be degraded by a β -elimination reaction in neutral and alkaline solutions (Fig. 16.7). The elimination reaction requires a methylated carboxyl group which assists removal of the proton from C-5 of the galacturonic acid residue. Keijbets and Pilnik (1974) found that β -elimination could occur when slightly acidic (pH 6.1) solutions of pectin were boiled for 30 min. Elimination was enhanced by the presence of both cations (Ca^{2+} , Mg^{2+}) and organic anions (citrate, malate, phytate) compared to K^+ and Cl^- . Similar changes in pectin were also observed when cell walls isolated from potatoes were boiled in pH 6.1 buffer (Keijbets *et al.* 1976). However, significant β -elimination was not observed when isolated cell walls from either potatoes or water chestnuts were heated in deionized water for shorter periods of time (≤ 5 min) at 90° – 110°C (Loh and Breene 1982). The heat treatments used were sufficient to cause considerable loss in fracturability of both potato and water chestnut tis-

sues. Though the pH, heating conditions, and salt concentrations in canned vegetables are such that β -elimination may occur during tissue softening, the reaction has not been directly demonstrated in processed vegetable tissues. For the most part, the specific chemical changes which result in softening of fruit and vegetable tissues during cooking have not been characterized.

The effects of calcium ion on tissue texture have been investigated in a variety of products and process conditions. There are many cases in which addition of calcium either increases the firmness of tissues or prevents loss of firmness. Perhaps the most common observation has been that fruit and vegetable firmness is improved by calcium addition after a mild blanch treatment. This type of firming has been found for frozen and canned snap beans, cauliflower, potatoes, tomatoes, cherries, apples (Van Buren 1979 and references), and carrots (Lee *et al.* 1979). The explanation of the firming effect is that blanching at 50°–80°C activates pectinesterase activity, pectin demethylation occurs, and calcium ions cross-link the demethylated pectin, which increases tissue firmness (Bartolome and Hoff 1972; Hoogzand and Doesburg 1961; Wiley and Lee 1970). However, Moledina *et al.* (1981) concluded that the firming effect of a precooking heat treatment of potatoes was related more to the increased availability of calcium ion released from the starch than to changes in pectin methylation caused by pectin methylesterase. Recently, a 12% increase in calcium binding by green bean components was observed when the beans were blanched at 71°C instead of 93°C prior to retorting (Van Buren 1980). This was attributed to an increase in the number of calcium binding sites after partial pectin demethylation.

The pectin in cucumber cell walls has been found to undergo extensive demethylation when the unheated fruit are fermented in a 6% salt brine with or without added calcium ion (Tang and McFeeters 1983). This demethylation is accompanied by an increase in mesocarp tissue firmness, which could be caused by pectin cross-linking after demethylation. However, when firmness was lost in the cucumbers without added calcium during storage, there were no significant changes in total pectin, pectin size, or noncellulosic neutral sugars in the isolated cell walls that correlated with firmness loss.

Recent studies in this laboratory on firmness changes and pectin methylation in blanched cucumber slices stored in acid brines (pH $\approx$ 3.7) show that calcium ion is very effective in maintaining tissue firmness (McFeeters *et al.* 1985). However, the effect of calcium is just as great with high methylation (40–50%) as low methylation (10–20%). Since at the storage pH most free carboxyl groups are also uncharged, conditions, particularly in the samples with high methylation, do not ap-

pear to be conducive to cooperative calcium cross-linking of the pectin to form egg box structures (Grant *et al.* 1973). These results suggest that it may be necessary to look for other mechanisms to explain some of the calcium firming effects that occur in acid products.

CELLULOSE STRUCTURE AND FUNCTION

Cellulose appears to provide the basic structural rigidity to the primary cell walls of all higher plants. The biosynthesis, structure, and degradation of cellulose has been extensively discussed in a recent book (Brown 1982). The polymer consists of linear chains of β -1,4-linked glucose residues which form both intra- and interchain hydrogen bonds to produce highly insoluble cellulose fibers (Fig. 16.8). In the cell walls of higher plants, cellulose molecules are arranged in microfibrils about 10 nm wide and 5 nm thick (Preston 1974). There are crystalline and amorphous regions within the microfibrils. The crystalline regions may be as long as 80–120 nm. Based upon X-ray diffraction studies of cellulose from the algae *Valonia*, it is believed that within the microfibrils, cellulose molecules have a parallel arrangement (Gardner and Blackwell 1974). Cellulose molecules have been found to contain from about 1000 (Thornber and Northcote 1962) (160,000 MW) to 44,000 glucose units (7,000,000 MW) for a high-molecular-weight cellulose fraction from *Valonia* (Palma *et al.* 1976).

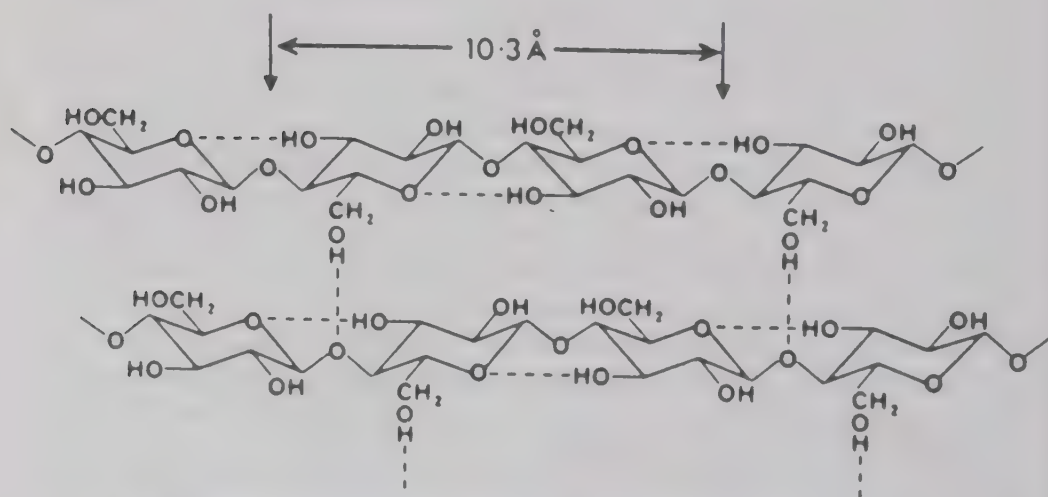


FIG. 16.8. The structural features of the β -($\rightarrow$ 4)-linked D-glucan chains of cellulose.

Adapted from Selvendran (1983).

Cellulose chains have an extended ribbon conformation, with two glucose residues repeating every 10.3 Å. Therefore, the largest cellulose molecules will be over 20 μm long. Considering the relative length of crystalline cellulose regions and the length of individual molecules, a single molecule may pass through many crystalline regions.

Most research on cellulose structure has been done using systems such as Valonia and cotton fibers, which produce large amounts of reasonably pure cellulose. Information on molecular size, proportion of crystalline and amorphous cellulose, and detailed microfibril structure has not been obtained on cellulose from fruit and vegetable tissues.

Approximately 20–60% of the cell wall polysaccharides from fruits and vegetables is cellulose. Generally, this cellulose contains 5–20% of sugars other than glucose, including xylose, arabinose, galactose, and uronic acid (Voragen *et al.* 1983; Selvendran 1983). It is not clear whether these sugars are covalently bound to cellulose or whether there are strong noncovalent attachments. O'Neill and Selvendran (1982) have reported that hydroxyproline-rich glycoproteins may be covalently cross-linked to cellulose through phenolic compounds.

Cellulose is much less reactive in foods than pectin, so there have been few investigations of changes in cellulose during processing and storage of foods. One area that has received some attention has been enzymatic degradation of cellulose by cellulases. The main interest in this research has been the development of economic processes for conversion of cellulose to ethanol via glucose (Wilke 1975; Gaden *et al.* 1976). Efforts to utilize cellulases in food processing have been primarily directed toward improving the extraction of cellular components. Examples include the isolation of starch (Toyama 1969) and extraction of olive oil (Fantozzi *et al.* 1977; Leone *et al.* 1977). There have also been attempts to use cellulases in the production of vegetable purees (Toyama 1969; Ghose and Pathak 1973).

Cellulase, which degrades carboxymethylcellulose but not crystalline cellulose, has been found to be present in tomatoes (Pharr and Dickinson 1973), peaches (Hinton and Pressey 1974), and mangoes (Roe and Bruemmer 1981). The cellulase activity increased during ripening in each of these fruits. However, the role of this cellulase activity in causing texture changes in the fruit has not been determined.

Degradation by reactive oxygen species is a reaction which cellulose shares with most, if not all, polysaccharides. Kon and Schwimmer (1977) added xanthine oxidase to polysaccharide solutions to generate superoxide anion and hydrogen peroxide, and then measured viscosity changes. Each polysaccharide tested, including methylcel-

lulose, starch, pectin, and guar gum, showed a 30–50% decline in viscosity, which suggested that degradation of the polysaccharide chains had occurred. The effect of inhibitors on the reaction indicated that degradation was caused by hydroxyl radical and singlet oxygen. They suggested that the potential exists for generation of reactive forms of oxygen in foods which may result in texture changes. This remains to be demonstrated in food systems. There have been recent investigations of possible mechanisms by which hydroxyl radicals can attack dextrans and pectins (Gilbert *et al.* 1984).

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Chemical Changes of Vitamins during Food Processing

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INTRODUCTION

Evaluation of the chemical effects of food processing on vitamins is a difficult task because of the diverse nature of the various vitamins as well as the chemical heterogeneity within each class of compounds. Although the general stability properties of many of the vitamins have been determined, many of the factors affecting retention of vitamins in complex food systems have not been well established. The variables that must be considered include (a) the time and temperature of processing or storage; (b) the concentration dependence and temperature dependence of the degradation reaction; (c) environmental variables such as the pH and the concentration of oxygen, metal ions, and various reducing or oxidizing agents; (d) the rate of competing or sequential reactions; (e) the relative stability of the various forms of a vitamin present; (f) the chemical nature of other food components (e.g., reducing vs. nonreducing sugars); (g) the mechanism(s) of chemical loss of vitamin activity in the food; and (h) the water activity of the food system. Because of the complex interactions of these variables, generalization on the basis of existing knowledge about the stability of vitamins in specific foods is often unreliable.

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In the context of this chapter, the term "processing" is used to include all aspects of food handling and chemical or physical preservation prior to consumption. Although losses of nutritional quality with respect to certain vitamins are often inevitable during food processing and storage, further elucidation of the mechanism and kinetics of the chemical reactions responsible would aid in selecting processing and storage conditions which could minimize these losses. Such studies of vitamin stability would also point out situations in which fortification would be warranted.

The objective of this chapter is to examine the chemical behavior of selected vitamins in relation to their stability and biological properties as influenced by food processing and storage. The major emphasis will be an evaluation of the primary types of processing and storage effects including (a) conversion of the vitamin to biologically inactive products; (b) conversion to a chemical form exhibiting reduced biological activity; (c) alteration of the chemical or physical form, which may increase or decrease the bioavailability of the vitamin; and (d) alteration of other food components which affect the biological activity or bioavailability of the vitamin. Because of the complexity of this topic, a comprehensive review is beyond the scope of this chapter. However, examples of each type of processing effect will be discussed. The interrelationships of vitamin chemistry and nutritional properties will be considered in each case.

VITAMIN DEGRADATION REACTIONS

The chemical conversion of vitamin compounds to biologically inactive products during food processing and storage has been the subject of extensive research. Although generalizations concerning vitamin stability have been attempted (e.g., Table 17.1; Harris 1971), many exceptions exist and invalid conclusions could be reached on the basis of these generalizations. The subject of vitamin stability has been discussed in many reviews which have dealt primarily with the kinetics of vitamin degradation and the relative stability of vitamins during thermal processing and storage of foods (Lund 1975, 1977, 1979; Kirk 1981; Karel 1979; Anderson *et al.* 1976; Cort *et al.* 1976; DeRitter 1976, 1982; Erdman 1979; Kramer 1974; Fennema 1977; Goldblith 1971; Mapson 1956; Chichester 1973). Most of these reviews have emphasized the rate and/or extent of loss of the vitamins and have not examined the mechanism of degradation or the identity of the products. The following is a summary of the vitamin degradation reactions which have been most thoroughly characterized. It is important to recognize that much of the information concerning these reactions

TABLE 17.1. GENERAL STABILITY OF VITAMINS

Nutrient	Effect of pH			Air or oxygen	Light	Heat	Maximum cooking losses (%)
	Neutral	Acid	Alkaline				
Vitamin A	S ^a	U	S	U	U	U	40
Ascorbic acid	U	S	U	U	U	U	100
Biotin	S	S	S	S	S	U	60
Carotenes	S	U	S	U	U	U	30
Choline	S	S	S	U	S	S	5
Vitamin B ₁₂	S	S	S	U	U	S	10
Vitamin D	S		U	U	U	U	40
Folic acid	U	U	S	U	U	U	100
Inositol	S	S	S	S	S	U	95
Vitamin K	S	U	U	S	U	S	5
Niacin	S	S	S	S	S	S	75
Pantothenic acid	S	U	U	S	S	U	50
Vitamin B ₆	S	S	S	S	U	U	40
Riboflavin	S	S	U	S	U	U	75
Thiamin	U	S	U	U	S	U	80
Tocopherols	S	S	S	U	U	U	55

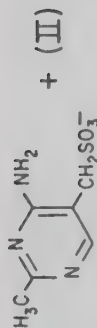
Adapted from Harris (1971).

^a S, Stable (no important destruction); U, unstable (significant destruction).

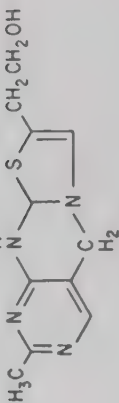
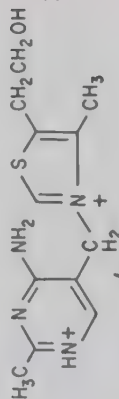
has been obtained using studies of vitamin compounds in defined solutions rather than actual foods. There is a distinct need for further study of degradation mechanisms, determination of the factors affecting the rate and extent of reaction, and the identification of reaction products in complex food systems.

Thiamin

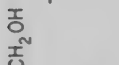
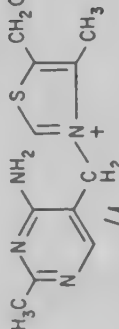
Because of its thermal instability in the neutral pH environment of many foods, the degradation of thiamin has been extensively studied. On the basis of research involving the effect of pH on the distribution of the various ionic/tautomeric forms of thiamin and pH effects on the pattern of the degradation products, two major pathways of the thiamin degradation have been proposed (Dwivedi and Arnold 1973). Heat treatment of thiamin solutions at pH 6.0 and below yields predominantly cleavage of thiamin at the methylene bridge between the substituted pyrimidine and thiazole moieties, with the formation of 2-methyl-4-amino-5-hydroxymethylpyrimidine and 4-methyl-5(β -hydroxyethyl) thiazole as major products. In contrast, thermal decomposition of thiamin at pH 7.0 or above yields hydrogen sulfide and various other sulfur-containing secondary degradation products of the thiazole ring. It was suggested that the activation energy of degra-



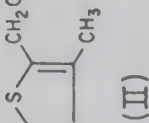
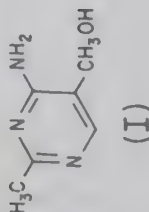
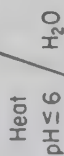
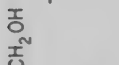
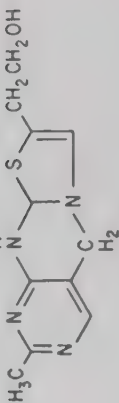
Thiamin (protonated)



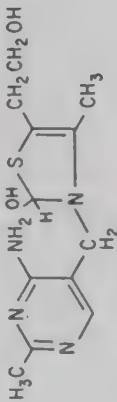
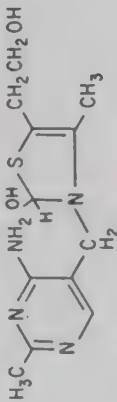
Thiamin (freebase)



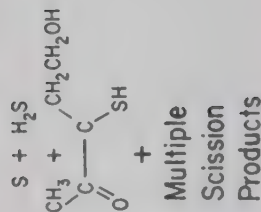
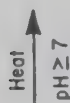
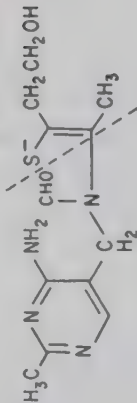
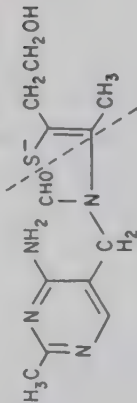
Thiochrome



Thiamin (pseudobase)



Thiamin (thiol form)



dation of the thiazole ring of the pseudobase and/or the thiol species of the thiamin, which predominate at $\text{pH} \geq 7.0$, is substantially lower than the activation energy for cleavage of the methylene bridge. Therefore, the degradation of thiamin via the thiazole breakdown mechanism would be favored at neutral or mildly alkaline pH, while at the slightly more acidic pH of most low-acid foods, the thermal degradation of thiamin would occur by both pathways. These interrelationships between degradation pathway, identity of the reaction products, and the ionic/tautomeric forms of thiamin are summarized in Fig. 17.1.

A limitation of many of the studies of thiamin degradation kinetics is the fact that thiamin pyrophosphate, the predominant naturally

TABLE 17.2. COMPARATIVE THERMAL STABILITY OF THIAMIN AND THIAMIN PYROPHOSPHATE IN 0.1 M PHOSPHATE BUFFER OF VARIED pH AT 265°C

Solution pH	Thiamin		Thiamin pyrophosphate	
	k^a (min^{-1})	$t_{1/2}$ (min)	k (min^{-1})	$t_{1/2}$ (min)
4.5	0.0230	30.1	0.0260	26.6
5.0	0.0215	32.2	0.0236	29.4
5.5	0.0214	32.4	0.0358	19.4
6.0	0.0303	22.9	0.0831	8.33
6.5	0.0640	10.8	0.1985	3.49

Adapted from Mulley *et al.* (1975).

^a k is the first-order rate constant and $t_{1/2}$ is the half-time for the thermal degradation reaction.

occurring form of thiamin in many foods, exhibits significantly less thermal stability than that of the nonphosphorylated compound. As shown in Table 17.2, the first-order rate constants for thiamin pyrophosphate degradation were from 1.12 to 3.27 times greater than those for thiamin, depending on the pH of the solution over the pH range 6.5–4.5 (Mulley *et al.* 1975). As a result, kinetic data derived from the analysis of thiamin degradation would underestimate the rate of

FIG. 17.1. Proposed major reactions involved in thiamin degradation. Thiamin degradation at pH values between 6 and 7 would involve both methylene bridge cleavage and thiazole ring cleavage pathways. Abbreviations: (I) 2-methyl-4-amino-5-hydroxymethylpyrimidine and (II) 4-methyl-5-(β -hydroxyethyl) thiazole. Dotted line through C–N bond of opened thiazole ring of thiol form of thiamin represents site of scission favored at $\text{pH} > 7$.

Modified from Dwivedi and Arnold (1973).

total thiamin loss in many foods. It is also important to recognize that the stability of thiamin during storage of low-moisture foods cannot be predicted by extrapolation from data derived from studies of thiamin in aqueous solution or high-moisture foods. Kinetic analysis of thiamin degradation in fortified cereals and model food systems indicated negligible losses of the vitamin during storage at water activity values of 0.10–0.65 at temperatures less than or equal to 37°C (Denison *et al.* 1977). Pronounced rate enhancement was observed at 45°C at all water activity values, although this would be of little significance in most food storage situations.

Most of the mechanistic and kinetic studies of thiamin degradation have been conducted using buffered solutions. An aspect of thiamin degradation which is not apparent in the previous discussions of thiamin chemistry is the potential effect of other food components on the rate and pathway of degradation. One example is the nucleophilic attack of bisulfite ions at the methylene bridge of thiamin, which induces bond cleavage and the formation of biologically inactive sulfonylpyrimidine and thiazole compounds (Metzler 1960). Several studies have shown that the extent of sulfite-mediated thiamin degradation in foods is directly proportional to the bisulfite concentration (Hermus 1969; Dwivedi and Arnold 1973). Although sulfiting agents are effective inhibitors of both nonenzymatic and enzymatic browning reactions of foods, the antithiamin activity of these compounds is a factor limiting their application in certain foods.

Another adverse effect of other food components on thiamin retention is the enhancement of thiamin degradation by hemin and heme proteins such as hemoglobin and myoglobin (Somogyi 1967; Kundig and Somogyi 1967; Porzio *et al.* 1973). Because of the fact that heme proteins exhibited antithiamin activity in both their native and denatured states, this rate-enhancing effect may be important in influencing the degradation of thiamin in the processing and subsequent storage of certain meat and seafood products. A variety of potential effects of proteins, carbohydrates, and metal ions on thiamin stability have been summarized in the review by Dwivedi and Arnold (1973). The role of tannins and other plant-derived phenolic compounds in enhancing the degradation of the thiamin in foods also has been demonstrated (Rungruangsak *et al.* 1977; Kositawattanakul *et al.* 1977; Yang and Pratt 1984). The biological significance of the tannin effects is not entirely clear at this time because one of the major products of this reaction, thiamin disulfide, retains thiamin activity (Yang and Pratt 1984). Ascorbic acid has been shown to inhibit the tannin-mediated degradation of thiamin (Rungruangsak *et al.* 1977). Overall, the main effects and possible interactions of many food components

on thiamin stability in complex food systems have not been evaluated sufficiently.

Vitamin B₆

The term "vitamin B₆" refers to a group of 2-methyl-3-hydroxy-5-hydroxymethylpyridine compounds. Depending on the nature of the single-carbon substituent group in the 4' position of the pyridine ring, the vitamin B₆ compound can be either an alcohol (pyridoxine, PN), an aldehyde (pyridoxal, PL), or an amine (pyridoxamine, PM). The presence of a phosphate group esterified to the 5' carbon yields pyridoxamine phosphate (PMP), pyridoxal phosphate (PLP), and pyridoxine phosphate (PNP). Although the six B₆ vitamers differ markedly in their thermal and storage stability properties, all of these compounds are highly susceptible to photochemical degradation (Reiber 1972; Ang 1979; Saidi and Warthesen 1983). The major product of photochemical degradation of PL and PLP, under conditions of excess available oxygen, has been found to be 4-pyridoxic acid, the biologically inactive carboxylic acid form of the vitamin, or its 5' phosphate (Morrison and Long 1958; Reiber 1972; Saidi and Warthesen 1983). The identity of the photolysis products of the other vitamin B₆ compounds has not been determined, but the product of photochemical degradation of PMP has been shown to be distinctly different from the 4-pyridoxic acid phosphate formed from PLP (Reiber 1972).

The differing relative stability properties of the various B₆ compounds have been of interest since the initial observation that the fortification of milk products with PN yielded greater retention of total vitamin B₆ activity than that observed for the naturally occurring B₆ compounds of milk (Hassinen *et al.* 1954). Navankasattusas and Lund (1982) examined the thermal stability of individual vitamin B₆ compounds in neutral phosphate buffer solutions and reported, as expected, that PN exhibited the greatest stability. Of interest was the fact that the apparent reaction order for the degradation reaction of each vitamer was concentration dependent and differed depending upon the particular vitamer being studied. Activation energy values for the thermal degradation of PM, PN, and PL were reported to be 85, 54, and 50 kcal/mol, respectively, under the conditions of their experiments. Research by Gregory and Hiner (1983) was conducted to examine the kinetics of the thermal degradation of PN, PM, PL, and PLP in simulated milk systems (pH 7.0) containing protein, sugars, ascorbic acid, and minerals (iron and zinc salts). Thermal losses of total vitamin B₆ from these model system solutions during retorting could be described by a first-order kinetic model, although the occur-

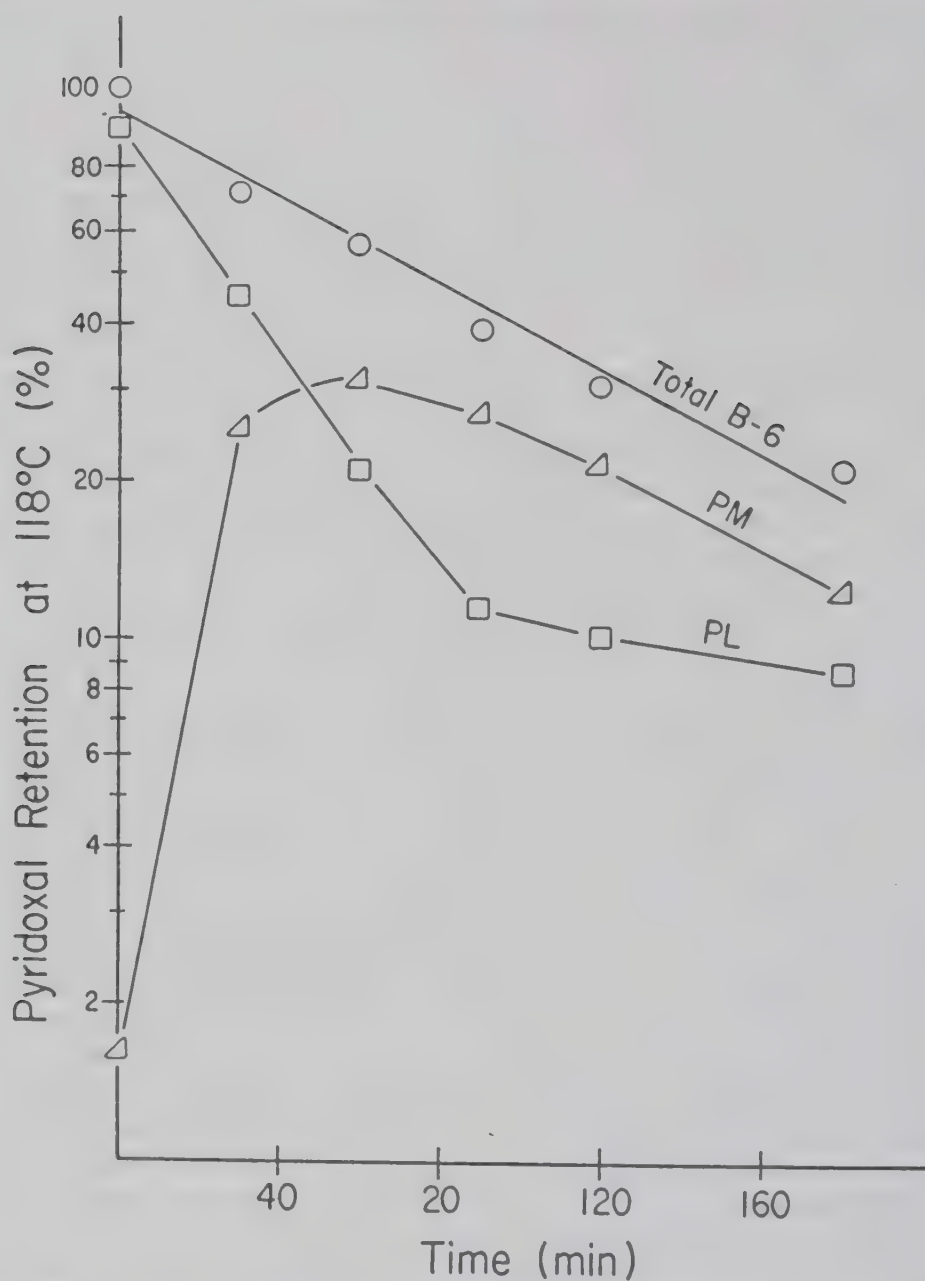


FIG. 17.2. Semilogarithmic plot of B₆ vitamers concentration during thermal processing time at 118°C in liquid model food system experimentally fortified with pyridoxal (PL) (from Gregory and Hiner 1983). Note the occurrence of transamination as indicated by the formation of pyridoxamine (PM).

rence of interconversions among all vitamers studied complicated the analysis of degradation kinetics of each form of the vitamin (Fig. 17.2). Data describing the thermal loss of total vitamin B₆ after enrichment of the model system solutions with individual B₆ vitamers are presented in Table 17.3. Activation energy values calculated for the loss of total vitamin B₆ following enrichment with either PM, PN, or PL were 23.7, 27.3, and 20.8 kcal/mol, respectively. The marked differences in activation energy observed by Gregory and Hiner (1983) and Navankasattusas and Lund (1982) are due presumably to environmental effects of the other ingredients and the fact that the B₆ vita-

TABLE 17.3. KINETIC DATA FOR THE LOSS OF TOTAL VITAMIN B₆ DURING RETORTING OF LIQUID MODEL SYSTEMS FORTIFIED WITH EITHER PYRIDOXINE, PYRIDOXAL, OR PYRIDOXAMINE

Vitamer added in fortification ^a	Processing temperature (°C)	First-order rate constant (min ⁻¹)	Half-life (min)
Pyridoxine	105	0.0006	1120
	118	0.0025	289
	133	0.0083	62
Pyridoxamine	105	0.0021	340
	118	0.0064	113
	133	0.0187	35
Pyridoxal	105	0.0040	179
	118	0.0092	75
	133	0.0266	24

From Gregory and Hiner (1983).

^a Liquid model systems (pH 7.0) contained potassium caseinate, sucrose, ascorbic acid, and minerals (zinc and iron) and were fortified with individual vitamin B₆ compounds.

mer concentrations used in the work by Navankasattusas and Lund were higher by approximately 80-fold. Factors such as the type of carbohydrate (reducing vs nonreducing sugar), ascorbic acid, and metal ions were found to affect the extent of degradation of certain B₆ vitamers, although the data indicated that vitamin B₆ retention was not strongly a function of food system composition (Gregory and Hiner 1983). Similar findings concerning the limited effects of other food components on vitamin stability have been reported by Navankasattusas (1978).

The relative thermal stability of PN, PL, PLP, and PM observed in the study of Gregory and Hiner (1983) was very similar to that observed in earlier kinetic studies of B₆ vitamer degradation during storage under reduced moisture conditions (water activity 0.6) (Gre-

TABLE 17.4. THERMODYNAMIC ACTIVATION PARAMETERS B_6 CALCULATED FROM PUBLISHED DATA DESCRIBING THE LOSS OF TOTAL VITAMIN B_6 DURING THERMAL PROCESSING OF SELECTED MODEL FOOD SYSTEMS AND CAULIFLOWER PUREE

Sample	Processing temperature (°C)	Vitamer added in fortification	Activation parameter ^a		
			$\Delta H^\ddagger$ (kcal/mol)	$\Delta S^\ddagger$ (e.u.)	$\Delta G^\ddagger$ (kcal/mol)
Liquid model system ^b	105	PN	27.6	-8.6	30.8
		PM	23.0	-18.4	29.8
		PL	20.0	-24.8	29.4
	118	PN	26.5	-11.3	30.9
		PM	22.9	-18.6	30.2
		PL	20.0	-25.3	29.9
	133	PN	26.5	-11.6	31.2
		PM	22.9	-18.8	30.5
		PL	20.0	-25.3	30.2
Dry model system ^c	155	PN	28.9	-7.8	32.3
	170	PN	28.9	-8.0	32.5
	185	PN	28.9	-8.0	32.5
	200	PN	28.9	-8.0	32.6
Cauliflower puree ^d	105.9	None	26.6	-8.6	29.9
	114.6	None	26.6	-9.2	30.2
	125.6	None	26.6	-10.6	30.8
	137.7	None	26.6	-11.5	31.3

^a Activation parameters: $\Delta H^\ddagger$, $\Delta S^\ddagger$, and $\Delta G^\ddagger$ refer to the apparent enthalpy, entropy, and free energy of activation, respectively.

^b Retorted liquid model system containing protein, sucrose, ascorbic acid, and minerals (Gregory and Hiner 1983).

^c Toasting of dehydrated model system containing protein, corn syrup solids, starch, and cellulose (Evans *et al.* 1981). Calculations based on first-order model for loss of PN during toasting.

^d Retorting of cauliflower puree (Navankasattusas and Lund 1982). Calculations based on pseudo-first-order degradation model with rate constants estimated over 1.5 hr heating time.

gory and Kirk 1978A), i.e., PN was markedly more stable than the other vitamers. In contrast, the relative stability of PN and two other vitamers (PM and PLP) was equivalent in studies of their behavior during high-temperature toasting of dehydrated food systems simulating breakfast cereal products (Gregory and Kirk 1978B). Subsequent kinetic studies of the degradation of PN during toasting, as with vitamin-fortified cereals, indicated that the loss of PN followed first-order kinetics (Table 17.4), with an activation energy of 29.8 kcal/mol (Evans *et al.* 1981). This value was in close agreement with the activation energy observed for the loss of PN in retorted liquid model systems (27.3 kcal/mol; Gregory and Hiner 1983).

Relatively little is known concerning the chemical mechanism of degradation of the B₆ vitamers and the identity of the degradation products. The first product to be tentatively identified was bis-4-pyridoxyl disulfide, which was found in milk that had been experimentally fortified with large amounts of PL before retort processing (Bernhart *et al.* 1960; Wendt and Bernhart 1960). This compound would presumably be formed via complexation of the PL with milk protein sulfhydryl groups, followed by dissociation of the B₆ vitamer as B₆ thiol derivatives which could react to form the bis-4-pyridoxyl disulfide product. Later studies of the sulfhydryl content of experimentally processed milk which had been fortified with large amounts of PL

1. Schiff base

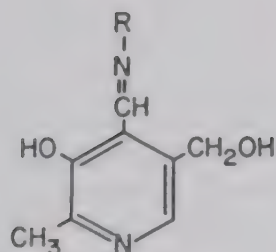
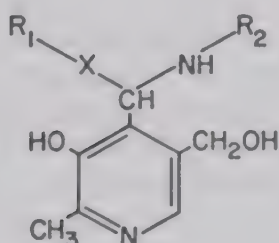
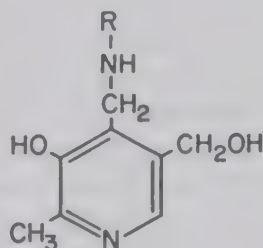
2. Substituted aldamine
(X=amino, sulfhydryl,
or imidazole)3. Pyridoxyl amino
compound

FIG. 17.3. Potential interactions involving pyridoxal and amino groups of food proteins. Pyridoxyl amino compounds would be formed by reduction of the Schiff-base azomethine linkage. Analogous reactions and interactions would occur with pyridoxal phosphate and proteins.

From Gregory and Kirk (1977).

supported the occurrence of the PL-sulfhydryl reaction (Srncova and Davidek 1972). Other research using PL and PLP levels comparable to those occurring naturally suggested that the reaction with protein sulfhydryl groups to form bis-4-pyridoxyl disulfide or other sulfur-containing products would not account for a significant proportion of the total loss of the vitamin during retort processing (J.F. Gregory, unpublished). Alternatively, the reductive bonding of PL and/or PLP to protein lysyl amino groups to form pyridoxyllysyl residues (Fig. 17.3) has been found to represent a significant mechanism for the loss of these vitamers in proteinaceous foods and liquid model systems during retorting and in dehydrated model systems during storage (Gregory and Kirk 1977, 1978A, 1981). The results of recent thermal

processing studies using radiolabeled forms of PL and PLP in liquid model systems simulating milk products as well as chicken liver and muscle tissue containing radiolabeled B₆ vitamers have confirmed that the reaction of B₆ vitamers with food proteins represents a major mechanism for losses encountered during thermal processing of proteinaceous foods (Gregory and Ink, unpublished). The nutritional properties of pyridoxyllysyl complexes will be discussed later. The extent of formation of protein-B₆ complexes other than as pyridoxyllysyl residues, analogous to the cyclic complexes of PL with cysteine, tryptophan, and histidine (Snell 1981), and the biological significance of these potential vitamin B₆ complexes in foods have not been examined. The only other product of vitamin B₆ degradation identified to date is 4-pyridoxic acid, which was found to be a minor product of PL degradation during the storage of low-moisture model food systems (Gregory and Kirk 1978A).

Whereas the mechanism of the thermal degradation of B₆ vitamers has not yet been fully determined, examination of the kinetics and apparent thermodynamic activation parameters provides qualitative information about the rate-limiting step. Constancy of the free energy of activation ($\Delta G^\ddagger$) is considered to be evidence against differences in reaction mechanism for structurally related compounds (Leffler 1955). Linearity of isokinetic plots of the activation entropy ($\Delta S^\ddagger$) vs the activation enthalpy ($\Delta H^\ddagger$) provides similar evidence for constancy of reaction mechanism among structural analogs (Leffler 1955, 1966; Labuza 1980). When this analysis was applied to published results concerning the thermal degradation of vitamin B₆ in liquid model systems, approximate constancy of apparent free energy of activation and linearity of isokinetic plots was observed (Table 17.4). These results provide indirect evidence for a common rate-limiting step in the degradation mechanism of PL, PN, and PM. The slopes of isokinetic plots were consistent with reaction rates being governed by the enthalpy of activation for each vitamer. Of further interest was the fact that apparent free energy of activation values calculated from previously published data concerning vitamin B₆ losses during the thermal processing of cauliflower puree (Navankasattusas and Lund 1982) and PN-fortified dry model systems (Evans *et al.* 1981) were in close agreement with values calculated from the kinetic data of Gregory and Hiner (1983). This is consistent with a common rate-limiting step in the degradation reaction(s) of the B₆ vitamers under widely differing conditions (Table 17.4). Further characterization of the degradation reactions is required for more conclusive interpretation of the thermodynamic data.

Ascorbic Acid

The stability properties of ascorbic acid vary markedly as a function of environmental conditions such as pH and the concentration of trace metal ions and oxygen. The chemistry of ascorbic acid degradation has been summarized in a review by Tannenbaum (1976) which emphasized the divergence of degradation mechanisms under aerobic and anaerobic conditions in the presence and absence of metal catalysts (Fig. 17.4). In general, the net stability of ascorbic acid increases in sigmoid fashion with decreasing pH because of the differing stability properties of the undissociated (fully protonated) and monoanionic species of the vitamin ($pK_{a1} = 4.04$). As would be predicted, the rate of oxidative degradation of ascorbic acid is proportional to the concentration of dissolved oxygen in the food system. The first product of ascorbic acid oxidation, dehydroascorbic acid, is biologically active as vitamin C. As a result, the reaction of greatest nutritional significance in the oxidative degradation of ascorbic acid is the hydrolytic decomposition of dehydroascorbic acid to form the biologically inactive 2,3-diketogulonic acid. The mechanism of the anaerobic degradation of ascorbic acid in foods is less well understood; however, it appears that 2,3-diketogulonic acid is formed by hydrolysis of the keto tautomeric forms of ascorbic acid (Fig. 17.4; Tannenbaum 1976). Further reactions in ascorbic acid degradation beyond 2,3-diketogulonic acid are of no nutritional consequence, but contribute to the flavor and color changes associated with browning reactions. The rates of metal-catalyzed ascorbic acid degradation reactions are often several orders of magnitude greater than those of the uncatalyzed reactions. Cupric copper has been found to be the most potent catalyst of ascorbic acid oxidation among the common metal ions in foods. Khan and Martell (1967) reported that cupric ion-catalyzed ascorbic acid oxidation is first order relative to oxygen concentration. In contrast to the stability properties of ascorbic acid in aerobic systems, the rate of anaerobic degradation of the vitamin is maximal when the pH equals the first pK_a (pH 4.0–4.1) (Lee *et al.* 1977). Ascorbic acid also exhibits sensitivity to photochemical degradation.

In addition to the effects of pH, oxygen, and metal ions, the stability of ascorbic acid has been found to be affected by other food components. Various sugars and their corresponding sugar alcohols have been shown to exert equivalent protective effects against the aerobic destruction of ascorbic acid (Birch and Pepper 1983). This action was attributed to the ability of carbohydrates to bind metal ions. In contrast, sugars have been shown to enhance the rate of anaerobic deg-

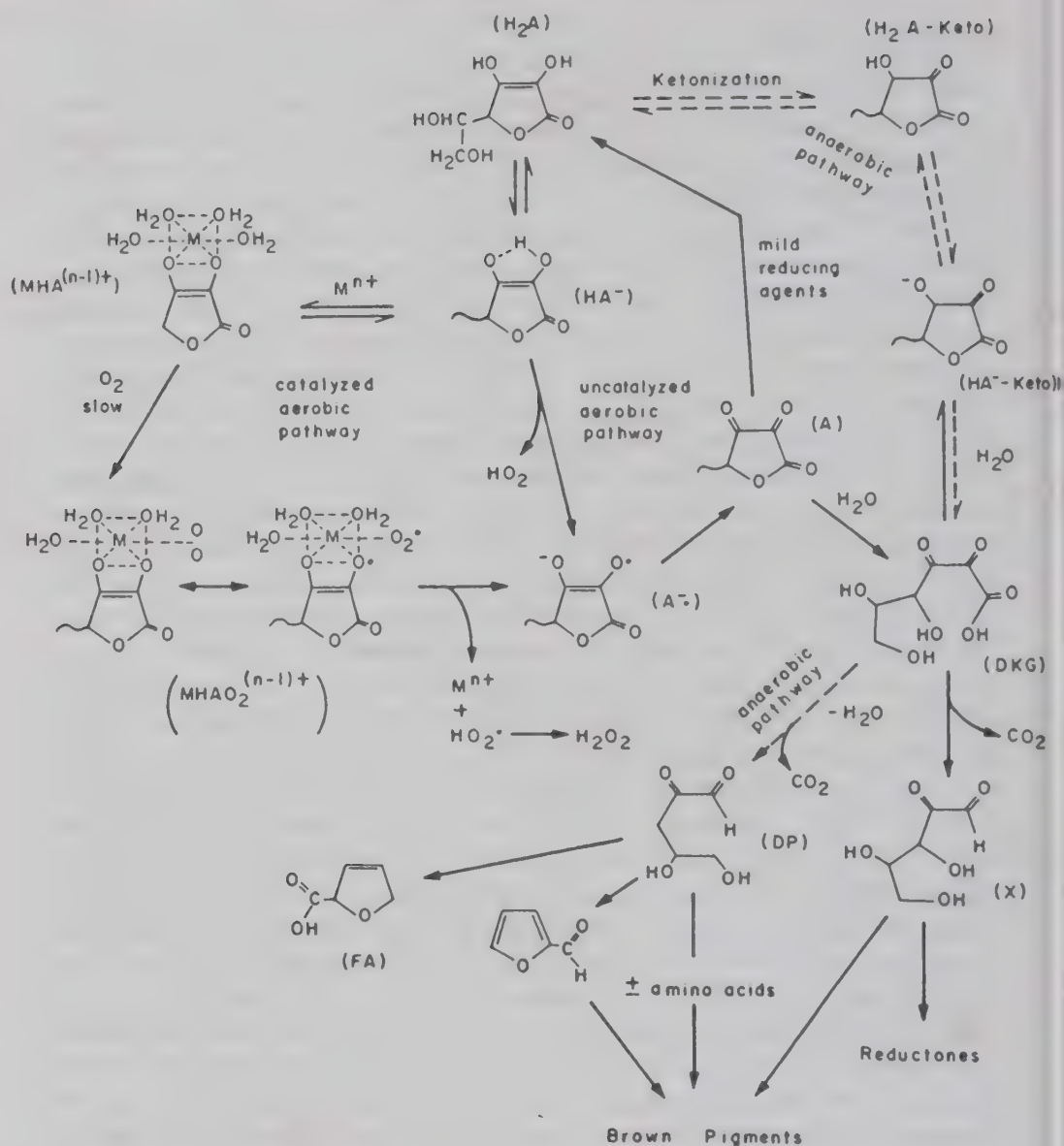


FIG. 17.4. Major reactions of ascorbic acid degradation. *Abbreviations:* Ascorbic acid, H_2A ; ascorbic acid monoanion, HA^- ; keto tautomers, H_2A -keto and HA^- -keto; dehydroascorbic acid, A; dehydroascorbate anion radical, $A^{\cdot-}$; metal ion catalyst, Mn^{n+} ; 2,3-diketogulonic acid, DKG; 3-deoxypentosone, DP; xylosone, X; 3,4-dihydro-2-furancarboxylic acid, FA.

From Tannenbaum (1976).

radation of ascorbic acid (Huelin 1953). Comparative studies of the anaerobic degradation of ascorbic acid in canned single-strength orange and grapefruit juices have demonstrated other effects of food composition (Smoot and Nagy 1980; Nagy 1980). In each juice product the degradation of ascorbic acid could best be described using a zero-order kinetic model. Studies of the temperature dependence of the reaction indicated that the Arrhenius plot for ascorbic acid degradation in canned single-strength grapefruit juice was linear over the range examined (4° – 50°C), while the Arrhenius plot for canned single-strength orange juice had a distinct transition suggesting a change in the degradation reaction at about 28°C . The energy of activation values for ascorbic acid degradation for orange juice were 12.8 Kcal/mol (4° – 28°C) and 24.5 kcal/mol (28° – 50°C), whereas that for grapefruit juice over the entire temperature range was 18.2 kcal/mol. These data demonstrate that subtle differences in food composition may have important effects on the rate and/or mechanism of ascorbic acid degradation.

In addition to the food composition variables mentioned, the nature of the packaging material significantly affects the stability of ascorbic acid in foods. Factors to be considered in examining the effect of packaging include the effectiveness of the material as a barrier to moisture and oxygen as well as the chemical nature of the surface exposed to the food product. Problems of ascorbic acid instability in aseptically packaged fruit juices have been encountered because of oxygen permeability and the oxygen dependence of the ascorbic acid degradation reaction. Because of the preferential oxidation of metallic tin, citrus juices packaged in cans with a tin contact surface exhibit greater stability of ascorbic acid than those in enameled cans or glass containers (Nagy 1980).

The aerobic and anaerobic degradation reactions of ascorbic acid in reduced-moisture foods have been shown to be highly sensitive to water activity. The reaction rate in many studies of low- to intermediate-moisture foods and model systems has been shown to increase in an exponential fashion over the water activity range ~ 0.1 – 0.8 (Waletzko and Labuza 1976; Warmbier *et al.* 1976; Riemer and Karel 1978; Mizrahi and Karel 1977A, B, 1978; Kirk *et al.* 1977; Dennison and Kirk 1978). This has been attributed to the role of water as a solvent in hydrating and mobilizing the reactants (ascorbic acid and oxygen) and metal catalysts. In studies of ascorbic acid degradation in model food systems, Dennison and Kirk (1978) observed that the activation energy for the loss of total (reduced + dehydroascorbic acid) ascorbic acid and dehydroascorbic acid increased as a function of increasing water activity (Table 17.5). Calculation of the thermodynamic acti-

TABLE 17.5 ENERGY OF ACTIVATION, RATE CONSTANTS, AND HALF-LIFE VALUES FOR THE LOSS OF REDUCED ASCORBIC ACID (RAA) AND TOTAL ASCORBIC ACID (TAA; REDUCED ASCORBIC ACID PLUS DEHYDROASCORBIC ACID) AS A FUNCTION OF WATER ACTIVITY DURING STORAGE OF A FORTIFIED DEHYDRATED MODEL FOOD SYSTEM OVER THE RANGE OF 10°–°C^a

Water activity	Activation energy (kcal/mol)		Kinetic parameters for TAA (30°C) ^a	
	TAA	RAA	<i>k</i> (days ⁻¹)	<i>t</i> _{1/2} (days)
0.10	8.1	7.1	0.0103	67
0.24	15.9	14.2	0.0363	19
0.40	17.6	16.8	0.0346	20
0.50	19.2	18.1	0.0328	21
0.65	19.2	19.3	0.0596	12

From Kirk et al. (1977).

^a *k* is first-order rate constant and *t*_{1/2} is the half-time for the degradation reaction.

vation parameters suggested that the dependence of activation energy on water activity did not reflect a change in reaction mechanism, but rather was due to an inverse relation between water activity and the entropy of activation ($\Delta S^\ddagger$) and a direct correlation between the enthalpy of activation ($\Delta H^\ddagger$) and water activity (Kirk and Dennison 1979; Kirk 1981). The decrease in entropy of activation with increasing water activity was interpreted as indicating greater ordering requirements in the solvation of reactants at low water activity.

Folacin

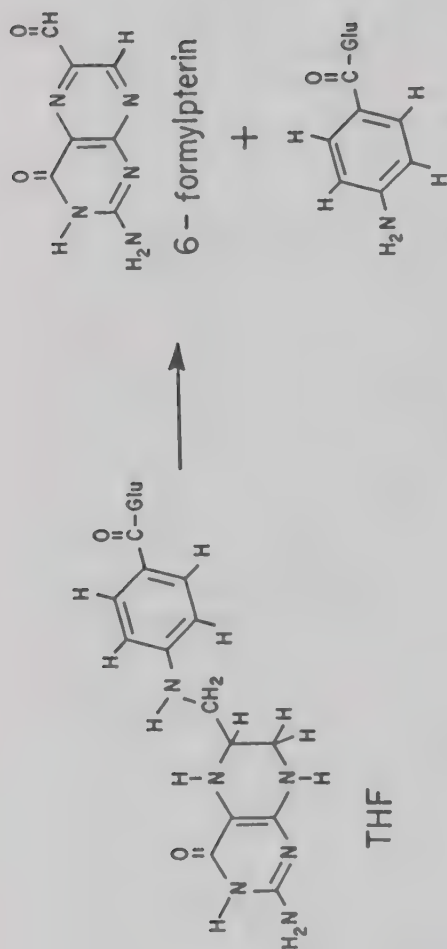
Because of the chemical diversity of the compounds that exhibit folacin activity (the biological activity qualitatively similar to that of folic acid), prediction of the retention of total folacin in foods is difficult. The folates differ widely with respect to their susceptibility to oxidative degradation, their thermal stability, and the pH dependence of their stability. Although the naturally occurring folates of plant and animal tissues exist predominantly in the form of folyl-polyglutamates of at least five glutamyl residues, most studies have shown that the length of the glutamyl side chain has little or no influence on the stability properties of the folacin compounds. In most naturally occurring folates the pteridine moiety is in the fully reduced 5,6,7,8-tetrahydro form. Single carbon substituent groups may occur at the N-5 or N-10 positions or as N-5–N-10 methenyl or methylene bridges. Folic acid does not occur naturally in appreciable quantities. The major naturally occurring folates in animal tissues are po-

lyglutamyl forms of tetrahydrofolic acid THF), 5-methyl-THF, and 10-formyl-THF, while polyglutamyl 5-methyl-THF comprises over 90% of the folates in milk (Gregory *et al.* 1984A). The folacin of plant tissues has been shown to be comprised mainly of polyglutamyl 5-methyl-THF, although THF has been found in significant quantities in many plant tissues (Gregory *et al.* 1984A).

The potential for large losses of folacin activity during the processing and home preparation of many foods has been well documented (Hurdle *et al.* 1968; Perloff and Butrum 1977; Huskinson and Retief 1970). In the commercial canning (blanching and retorting) and home cooking of vegetables a great deal of the loss of folacin appears to occur via leaching into the aqueous medium (Leichter *et al.* 1978; Miller *et al.* 1973; Lin *et al.* 1975).

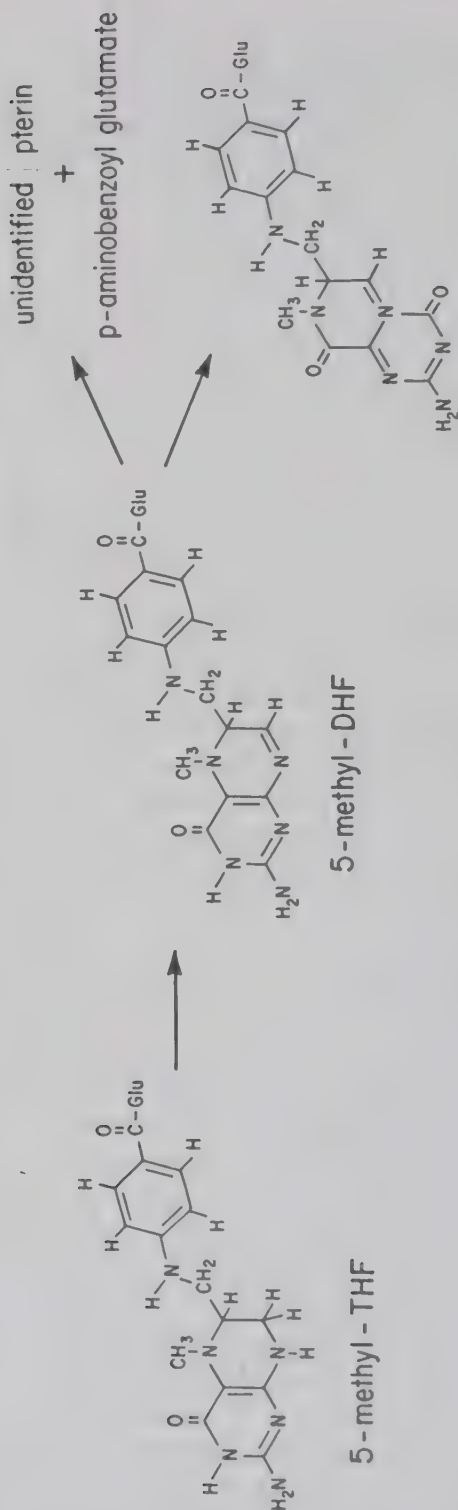
The most stable of the various folates at ambient and elevated temperature is folic acid; consequently, synthetic folic acid is the sole folacin compound used in food fortification (O'Broin *et al.* 1975). Tetrahydrofolic acid is extremely susceptible to oxidative cleavage of the C-9-N-10 bond to form biologically inactive pterin and *p*-aminobenzoyl glutamate products, as shown in Fig. 17.5A (Blair and Pearson 1974; Reed and Archer 1980). In contrast to the behavior of the other reduced folates, THF is less stable under alkaline conditions than in acid media (O'Broin *et al.* 1975). Folic acid is much more resistant to oxidative cleavage than THF. The presence of substituent groups in the N-5 and N-10 positions substantially increases the oxidative stability of the reduced folates relative to that of THF. The presence of reducing agents including thiols (e.g., mercaptoethanol) and ascorbic acid dramatically improves the stability of the folacin compounds (O'Broin *et al.* 1975), presumably by being preferentially oxidized and scavenging molecular oxygen. Although chemical procedures for the reductive cleavage of the C-9-N-10 bond of certain folates have been used analytically, such reactions do not appear to be involved in the degradation of folacin compounds in foods.

The oxidative behavior of 5-methyl-THF and 10-formyl-THF, while not fully determined in foods, can be predicted on the basis of the chemical nature of these compounds. In the presence of molecular oxygen or other oxidants, 5-methyl-THF, which is the principal naturally occurring form of folacin in many foods, undergoes partial oxidation to form 5-methyl-5,6-dihydrofolic acid (5-methyl-DHF; Fig. 17.5B) (Larrabee *et al.* 1961, 1963; Scrimgeour and Vitols 1966; Gupta and Huennekens 1970; Blair *et al.* 1975). 5-Methyl-DHF has been shown to exhibit folacin activity similar to that of folic acid (Gregory *et al.* 1984B), although it is highly susceptible to cleavage of the C-9-N-10 bond in strongly acidic media (Maruyama *et al.* 1978; Lewis



p-aminobenzoyl glutamate

A



B

5-methyl dihydropyrazino-s-s-triazine compound

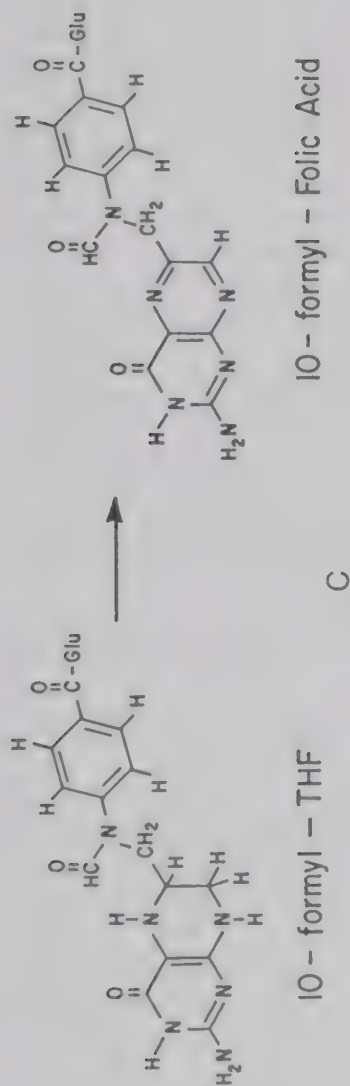


FIG. 17.5. Mechanisms for the oxidation of the principal naturally occurring folacin compounds. (A) Oxidative cleavage of tetrahydrofolic acid (THF). (B) Oxidation of 5-methyl-THF to 5-methyl-DHF, followed by its cleavage or rearrangement to form biologically inactive products. (C) Oxidation of 10-formyl-THF to 10-formylfolic acid (which retains biological activity).

and Rowe 1979). In mildly acidic media 5-methyl-DHF undergoes rearrangement of the pteridine ring system to a pyrazino-s-triazine form (Fig. 17.5B; Jongehan *et al.* 1979) which is biologically inactive (Gapski *et al.* 1971; Ratanasthien *et al.* 1977; Kennelly *et al.* 1982). The kinetics and environmental factors favoring the formation of the pyrazino-s-triazine have not yet been determined. The mechanism of oxidative degradation of 10-formyl-THF in foods also has not been fully elucidated. Oxidation of 10-formyl-THF yields 10-formylfolic acid (Fig. 17.5C; Maruyama *et al.* 1978; Lewis and Rowe 1979). 10-Formylfolic acid exhibits stability comparable to that of folic acid, although it has been shown to be degraded chemically by oxidative cleavage yielding pterin and *N*-formyl-*p*-aminobenzoyl glutamate fragments (Maruyama *et al.* 1978; Lewis and Rowe 1979).

Several kinetic studies have been conducted to examine the thermal stability of various folates in liquid model systems and buffered solutions (Paine-Wilson and Chen 1979; Chen and Cooper 1979; Ruddick *et al.* 1980; Mnkeni and Beveridge 1982, 1983; Day and Gregory 1983). Most of these studies have indicated that the degradation of various folates can be described by a first-order kinetic model, although the demonstrated dependence of reaction rate on oxygen concentration indicates that the reaction is pseudo-first order (Ruddick *et al.* 1980; Chen and Cooper 1979; Day and Gregory 1982). The reported activation energy values for thermal degradation of folacin compounds have varied widely (Chen and Cooper 1979; Ruddick *et al.* 1980; Mnkeni and Beveridge 1982, 1983), which presumably reflects differences in the temperature dependence of the reactions as a function of environmental conditions and oxygen concentration.

As with other vitamins, the chemical environment influences the rate of the degradation of folates. As previously indicated, the pH of the medium strongly influences folacin stability. Phosphate ions have been shown to accelerate the degradation of certain folates (O'Broin *et al.* 1975; Paine-Wilson and Chen 1979). Studies by Day and Gregory (1983) indicated that all ingredients of a liquid model milk system which were capable of lowering the concentration of dissolved oxygen, including casein, lactose, ferrous iron, and ascorbate, increased the thermal stability of folic acid and 5-methyl-THF. Of interest was the fact that the effects of iron and ascorbate were significant even at the low concentrations employed in the nutritional fortification of infant formula products. A similar interrelationship between oxygen, low levels of ascorbic acid, and 5-methyl-THF has been shown in the heat sterilization of milk (Ford 1967). Adverse effects of nitrite ions on the stability of various folates in dilute aqueous solution have been reported. Nitrite ions reacted with the folate com-

pounds to yield nitrosated folates, which may lack folacin activity, as well as various C-9–N-10 bond cleavage products (Reed and Archer 1979). Oxidizing agents used in certain foods, including hypochlorous acid, yield rapid oxidation and oxidative cleavage of many folates (Day and Gregory 1981). Few data are available concerning other influences of food composition and relations to folacin stability.

CONVERSION TO PRODUCTS EXHIBITING REDUCED BIOLOGICAL ACTIVITY

Chemical conversion of vitamins to products having lower nutritional activity than the parent compounds would significantly lower the nutritional quality of the foods involved. Whereas the degradation of the parent vitamin compounds can be conveniently monitored in most cases, relatively few analytical methods have been developed for the quantitation of vitamin reaction products exhibiting reduced vitamin activity. The formation of such compounds almost inevitably leads to inaccuracy in the evaluation of the vitamin nutritional quality. The following is a discussion of several examples of the formation of chemically altered forms of certain vitamins during food processing or storage which yields a reduction in the vitamin activity.

Stereoisomerization of Vitamin A and Carotenes

Dietary vitamin A activity for humans and many animal species is obtained both as retinol (and its esters) and as biologically active carotenes. The carotenes having provitamin A activity, of which β -carotene exhibits the greatest vitamin A activity, are metabolically inactive in their intact state. Their vitamin A activity arises from their enzymatic cleavage to form retinaldehyde catalyzed by intestinal β -carotene-15,15'-dioxygenase. The conjugated double-bond system of both retinol and its esters and carotenes gives rise to a number of possible stereoisomeric forms. All-trans forms of both retinol and carotenes exhibit greater vitamin A activity than their corresponding cis isomers (Tables 17.6 and 17.7). Any conversion of all-trans forms to cis isomers would, therefore, cause a reduction in the net vitamin A activity of the food product which could not be detected using traditional colorimetric methods for vitamin A and carotene quantitation.

Relatively few quantitative studies have been conducted to characterize the kinetics and factors affecting the isomerization reactions occurring during the thermal processing of foods. Losses of 15–35% of the vitamin A activity of vegetables have been reported for typical

TABLE 17.6. RELATIVE VITAMIN A ACTIVITY FOR RATS OF STEREOISOMERIC FORMS OF RETINOL DERIVATIVES

Isomer	Relative vitamin A activity ^a	
	Retinyl acetates	Retinaldehydes
All-trans	100	91
13-cis	75	93
11-cis	23	47
9-cis	24	19
9,13-di-cis	24	17
11,13-di-cis	15	31

From Ames (1965).

^a Molar vitamin A activity relative to that of *all-trans*-retinyl acetate based on rat growth and/or liver storage bioassay methods.

TABLE 17.7. RELATIVE VITAMIN A ACTIVITY FOR RATS OF STEREOISOMERIC FORMS OF CAROTENE

Isomer	Relative vitamin A activity ^a
<i>β</i> -Carotenes	
All-trans	100
9-cis (neo-U)	38
9,13-di-cis (neo-B)	53
<i>α</i> -Carotenes	
All-trans	53
9-cis (neo-U)	13
9,13-di-cis (neo-B)	16

From Zechmeister (1949).

^a Activity relative to that of *all-trans-β*-carotene in rat growth bioassay.

thermal processing and home-cooking procedures as a result of carotene stereoisomerization (Sweeney and Marsh 1970, 1971; Ogunlesi and Lee 1979). Similar isomerization has been detected for retinol and its esters in various food products (Egberg *et al.* 1977; Mulry *et al.* 1983). With the development of improved chromatographic methods for the separation and quantitation of vitamin A and carotene stereoisomers, the rate and extent of formation of *cis* isomers and their nutritional significance will be more fully determined.

Vitamin E

During the oxidative degradation of *α*-tocopherol, the formation of a variety of products has been reported. These include *α*-tocopheryl

oxide, α -tocopheryl quinone, and α -tocopheryl dimer and trimer (Csallany *et al.* 1969; Widicus *et al.* 1980; Widicus and Kirk 1981). Widicus (1980) evaluated several tocopherol oxidation products for their vitamin E activity using the specific rat bioassay method of Machlin *et al.* (1978) which is based on the rise in plasma pyruvate kinase activity during vitamin E deficiency. On the basis of this measure of biological activity, α -tocopherol oxide, α -tocopheryl quinone, and α -tocopheryl dimer exhibited approximately 127, 35, and 7% of the molar potency of α -tocopherol. These results suggest that certain oxidation products of tocopherols may retain at least partial biological activity which would not be measurable in conventional methods of vitamin E determination.

Binding of Vitamin B₆ Aldehydes to Food Proteins

The reaction of the aldehyde group of pyridoxal (PL) or pyridoxal phosphate (PLP) with proteins during food processing has been shown to account for at least a portion of the losses of vitamin B₆ reported in proteinaceous foods as a result of thermal processing or storage (Register *et al.* 1950; Tomarelli *et al.* 1955; Harding *et al.* 1959). As previously discussed, interaction of PL or PLP with protein sulfhydryl groups has been reported to yield bis-4-pyridoxyl disulfide as a product (Bernhart *et al.* 1960; Wendt and Bernhart 1960); however, the reductive bonding of these B₆ compounds to amino groups which yields pyridoxyl amino compounds such as pyridoxyllsine appears to be a more quantitatively significant reaction (Gregory and Kirk 1977, 1978A; Gregory and Ink, unpublished).

Studies of the biological activity of bis-4-pyridoxyl disulfide in rats indicated that it exhibited 12–23% molar vitamin B₆ activity compared to that of pyridoxine, based on rat growth and 20% molar activity in the standard microbiological assay for vitamin B₆ (Bernhart *et al.* 1960). No evidence of antivitamin activity of bis-4-pyridoxyl disulfide was obtained in the rat experiments, although biochemically specific indicators of vitamin B₆ nutritional status were not evaluated. Extensive studies of the biological activity of dietary protein-bound pyridoxyllsine (as phosphopyridoxyl bovine serum albumin, PPBSA) have been conducted. On the basis of rat growth and liver PLP concentration as bioassay criteria, the pyridoxyllsyl residues of PPBSA exhibited ~50% molar vitamin B₆ activity relative to pyridoxine (Gregory and Kirk 1978C; Gregory 1980A). When fed at low levels to vitamin B₆-deficient rats, PPBSA increased the severity and rate of onset of overt vitamin B₆ deficiency symptoms. This effect was also reflected in enzymatic assessment of vitamin B₆ nutriture of the rats

(Gregory 1980A). The antagonistic activity could be overcome by the addition of small amounts of pyridoxine to the diets. At higher dietary levels of PPBSA, no antivitamin effect was observed. Biochemical studies indicated that pyridoxyllysine was metabolized to form PLP via the same pathway as the free B₆ vitamers, while the antivitamin activity may have been due to a competitive inhibition of normal B₆ metabolism and/or a direct anticonzyme effect of pyridoxyllysine or phosphopyridoxyllysine (Gregory 1980B). Under normal conditions in humans or animals, the antivitamin activity of this complexed form of the vitamin would have little or no nutritional significance under most circumstances. It may be speculated that this antivitamin B₆ effect may have been involved in the severe vitamin B₆ deficiency exhibited by infants fed severely heated nonfortified infant formulas in the early 1950s (Coursin 1954).

The objective of most food analyses for the determination of vitamin B₆ and other nutrients is to obtain a numerical value which ideally reflects the biological activity of the nutrient in the food. The existence of protein-bound forms of vitamin B₆ may complicate this interpretation. Much of the vitamin B₆ of animal-derived foods exists as PLP bound to protein as reversible Schiff-base forms which readily dissociate during the extraction. In contrast, vitamin B₆ as pyridoxyllysyl residues of food proteins would not be dissociated under typical extraction conditions. Thus, the determination of vitamin B₆ by conventional assay methods would not account for the biological activity of protein-bound pyridoxyllysyl residues even though free pyridoxyllysine (not bound to protein) yields a full molar response in the microbiological assay (Gregory and Kirk 1981). Further research is needed to evaluate the extent of this problem and its nutritional significance. Relatively little is known concerning the possible existence of analogous situations involving other vitamins.

Thiamin

Evidence of the binding of thiamin to protein via a mixed disulfide linkage during thermal processing has been reported (Morfee and Liska 1971). Although the identity of the complex was not determined conclusively, the formation of a mixed disulfide complex analogous to thiamin disulfide appears likely. The disulfide-linked form of thiamin would not be measurable in standard chemical assay procedures unless a disulfide reducing agent were incorporated. Because thiamin disulfide exhibits full thiamin activity via its facile nonenzymatic reduction (Evans 1975), human or animal diets containing disulfide forms

of thiamin would probably exhibit thiamin activity that would be measurable in routine chemical assays.

FOLACIN

As previously discussed, the oxidative cleavage of folates during food processing, preparation, or storage yields biologically inactive products. Partial oxidation of naturally occurring folates such as in the formation of 5-methyl-DHF and 10-formylfolic acid has been reported to yield little or no loss of folacin activity (Gregory *et al.* 1984B). Although 10-formylfolic acid is a fairly stable compound, 5-methyl-DHF is quite acid labile. As a result, the biological activity of 5-methyl-DHF would be influenced by factors affecting its stability in the acidic gastric environment. In this context, the buffering action of foods would tend to increase the stability of 5-methyl-DHF against acid-induced cleavage of the C-9–N-10 bond, while unbuffered oral doses of 5-methyl-DHF probably would undergo significant degradation. Apart from C-9–N-10 bond cleavage, 5-methyl-DHF also may be converted to a biologically inactive product by rearrangement of the 5-methyldihydropteridine moiety of the molecule to form a pyrazino-s-triazine in mildly acidic media, as previously discussed. These degradation reactions of 5-methyl-DHF are summarized in Fig. 17.5B. The effects of the chemical environment on the rate of conversion of 5-methyl-DHF to the pyrazino-s-triazine compound have not yet been determined, although the extent of this reaction in the gastric contents represents a possible influence of diet composition on the net *in vivo* biological activity of 5-methyl-DHF.

PROCESSING EFFECTS ON VITAMIN BIOAVAILABILITY

In contrast to the term “biological activity,” which refers to the inherent potency of a compound in meeting a specific nutritional requirement, “bioavailability” refers to the extent of intestinal absorption and metabolic utilization of a nutrient. Consequently, the bioavailability of a nutrient could be influenced by its chemical form or physical state as well as by other dietary components that could affect its absorption and metabolism. The following is a discussion of several examples of alterations of vitamin bioavailability as related to food processing. It must be emphasized that the nature of dietary, environmental, and processing factors affecting vitamin bioavailability is very poorly understood at this time.

Niacin Bioavailability

The occurrence of niacin in many cereal grains as various conjugated derivatives has long been known to be a major factor influencing its bioavailability. Although the chemical nature of the conjugated forms has not been identified unequivocally, isolation and analysis of niacin complexes from several cereal grain materials have been reported (Das and Guha 1960; Kodicek and Wilson 1960; Christianson *et al.* 1968; Mason *et al.* 1973). The composition of the isolated niacin conjugates varied as a function of the source; however, all exhibited a broad molecular weight range and contained variable amounts of covalently bound sugars, peptides, and phenolic compounds. Chromatographic analyses revealed a high degree of heterogeneity within preparations from both wheat bran and corn (Christianson *et al.* 1968; Mason *et al.* 1973).

The results of many animal bioassays have suggested that cereal grain products and isolated conjugated forms of niacin exhibit little or no biologically available niacin activity, while alkaline treatments convert the "bound" niacin to an available form (Kodicek 1960; Carpenter *et al.* 1960; Chaudhuri and Kodicek 1960; Christianson *et al.* 1968). These observations were of particular significance with respect to the alkaline treatment of various corn products. This property also is of analytical importance because measurement of *total* niacin in a food would require an alkaline extraction step in order to release conjugated forms of niacin. Several authors have suggested that the measurement of "free" (0.1 *N* HCl extractable) niacin in foods would provide an accurate estimate of the content of biologically available niacin. However, recent studies by Carter and Carpenter (1982) suggest that the measurement of only free niacin may yield an underestimation of available niacin because of the apparent partial utilization of the conjugated niacin by rats in their bioassays. Results of chemical and biological assays of various foods for total and biologically available niacin from the Carter and Carpenter (1982) study are presented in Table 17.8.

Vitamin B₆ Bioavailability

The effects of food processing on the apparent bioavailability of vitamin B₆ have been examined extensively as a result of reports in the 1950s concerning the bioavailability of vitamin B₆ in retorted milk and infant formula products and military rations (Register *et al.* 1950; Tomarelli *et al.* 1955). As discussed previously, these effects may be due to reduced vitamin activity of complexes formed during process-

ing and/or antagonistic effects such as that described for pyridoxyllysine (Gregory 1980A).

Another interesting example that may be related to processing conditions and food composition is the bioavailability of vitamin B₆ in breakfast cereals fortified with pyridoxine. It would be expected that the pyridoxine added in fortification would be fully available in all cases because of the chemical inertness of this form of the vitamin. This high bioavailability has been shown in studies of pyridoxine in dehydrated model food systems during storage and high-temperature toasting (Gregory and Kirk 1978A, B). However, analysis of a com-

TABLE 17.8. CONCENTRATION OF TOTAL AND BIOLOGICALLY AVAILABLE NIACIN IN SELECTED FOODS

Food material	Total niacin ^a (mg/kg)	Available niacin ^b (mg/kg)	Apparent bioavailability (%)
Wheat bran concentrate	2830.0	480	17
Whole wheat	51.4	16	31
Rice, boiled	70.7	29	41
Corn, raw	28.0	7	25
Corn, baked	27.0	11	40
Potatoes, baked	50.7	32	63
Peanut flour	255.0	117	46

From Carter and Carpenter (1982).

^a Determined by chemical analysis using alkaline hydrolysis.

^b Determined by rat bioassay. Values include niacin activity derived from dietary tryptophan.

mercial rice-base breakfast cereal product for total and biologically available vitamin B₆ indicated incomplete utilization of the vitamin in the rat bioassay (Gregory 1980C). In contrast, a similar fortified corn-base cereal product exhibited full bioavailability of the vitamin under similar assay conditions (J.F. Gregory, unpublished). As summarized in Table 17.9, these results suggest that the composition and physical form of the food and the type and extent of thermal processing in addition to the chemical behavior of the vitamin significantly affect the bioavailability of vitamin B₆ in foods.

Effects of Thermal Processing on Antagonistic Properties of Food Proteins

The apparent bioavailability of vitamins can be influenced by dietary components which either alter their intestinal absorption or their

TABLE 17.9. CONCENTRATION OF TOTAL AND BIOLOGICALLY AVAILABLE VITAMIN B₆ IN SELECTED BREAKFAST CEREAL PRODUCTS^a

Assay method	Cereal product base	
	Corn ($\mu\text{g B}_6/\text{g}$)	Rice ($\mu\text{g B}_6/\text{g}$)
Available vitamin B ₆ ^b		
Rat growth	35.6	15.1
Feed efficiency	33.5	9.3
AspAT activity	35.5	15.0
Liver PLP	—	6.2
Total vitamin B ₆		
Microbiological assay	36.5	33.9
HPLC	34.7	35.5

^a Data for corn-base cereal unpublished (J.F. Gregory). Data for rice-base cereal from Gregory (1980).

^b Available vitamin B₆ in cereals determined by rat bioassay using the following response criteria: rat growth, feed efficiency (growth/gram of feed consumed), erythrocyte aspartate aminotransferase (AspAT) activity, and liver pyridoxal phosphate (PLP) concentration.

in vivo metabolism. As a result, chemical changes in the properties of other dietary components during thermal processing indirectly may affect the apparent bioavailability of certain vitamins. This has been shown in several cases involving various types of dietary proteins, although the mechanism of action has not been determined adequately in many cases.

Probably the best example of an inhibitory effect of a food protein on the bioavailability of a vitamin is that of the adverse effect of raw egg albumen on biotin utilization. As reviewed by Osuga and Feeney (1977), avidin, which is a minor protein in egg albumen, specifically binds biotin with a dissociation constant of 10^{-15} M. This complex dissociates extremely slowly and is resistant to proteolytic degradation such that dietary biotin in the presence of avidin exhibits little or no biological availability. Heat treatment of egg protein to denature avidin eliminates its inhibitory properties.

The first reported instance of an antagonistic effect of raw soybean meal on the apparent bioavailability of a vitamin involved an increase in the blood clotting time of chicks fed diets containing raw soybean meal as compared to diets containing autoclaved soybean meal (Balloun and Johnson 1953). Because of the increase in clotting time associated with vitamin K deficiency, this was suggestive of an impaired utilization of dietary vitamin K induced by a heat-labile factor. The fact that the anticoagulant effect of the raw soybean meal was not reversed by supplemental vitamin K suggests, however, that

the anticoagulant properties were not mediated by inhibition of vitamin K absorption or metabolism (Balloun and Johnson 1953).

Similar observations were reported in the case of the rachitogenic properties of various soybean protein fractions in experimental poultry diets (Carlson *et al.* 1964; Thompson *et al.* 1968). In these studies supplemental vitamin D₃ tended to normalize growth, bone mineralization, serum calcium, and alkaline phosphatase activity values, although dietary supplementation with vitamin D₃ even at ten times the National Research Council requirement did not fully overcome the rachitogenic effects of raw soy protein isolate (Carlson *et al.* 1964). Autoclaving of the soy protein isolate at 120°C for various times up to 60 min yielded progressively lower rachitogenic properties (Thompson *et al.* 1968). It could not be determined from these studies whether the adverse effect of the heat-labile soy component was mediated via direct impairment of the utilization of dietary vitamin D.

On the basis of early observations that the addition of vitamin B₁₂ improved the growth of rats and chicks that were fed raw soybean fractions, research was conducted to examine the mechanism involved. Preliminary experiments with rats confirmed that raw soy flour induced a vitamin B₁₂ deficiency which was fully reversible by the addition of vitamin B₁₂ or substitution of heated soy flour for the raw flour (Edelstein and Guggenheim 1969). Subsequent studies suggested that the vitamin B₁₂ deficiency was induced by an impairment in the utilization of B₁₂ synthesized by the intestinal microflora, as well as an increased *in vivo* turnover of the vitamin (Edelstein and Guggenheim 1969). As in the case of the other antinutritional properties of raw soy fractions, these effects were reduced or eliminated by heat treatment. These findings, along with those concerning the rachitogenic and anticoagulant effects of raw soybean fractions, suggest that the protease inhibitors, lectins, and other heat-labile antinutritional factors affect intestinal homeostasis such that the apparent bioavailability of a variety of vitamins may be affected.

CONCLUSIONS

A great deal has been learned concerning the chemistry of vitamin degradation; however, the complexity of food processing effects on vitamins is such that our present level of understanding is largely inadequate. As indicated throughout this chapter, much of the uncertainty lies in understanding the interactive effects of other food components on the rate, temperature dependence, and pathway of vitamin degradation reactions. The chemical nature and nutritional

properties of vitamin degradation products are another important area in which the basic chemistry and environmental effects have not been clearly determined. In addition, vitamin bioavailability, especially as influenced by food processing, is very poorly understood at this time. Clarification of the complex chemical and physical processes involved in the behavior of vitamins will greatly enhance our understanding of the nutritional quality of foods.

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Chemical Changes in Natural Food Pigments

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INTRODUCTION

This symposium is structured around the chemical changes in food processing. While some of these changes are beneficial, most result in lowered quality. Nowhere is a change more apparent than one involving a change in pigmentation. This may result in a change in intensity as in bleaching of a food or a hue change resulting in off-color. Even if the color change is the only change, that difference could be sufficient for consumer rejection. Color also plays a role in product identification. Fabricated products, soda, candies, etc., and natural products such as trout and salmon are identified on the basis of color even though color affects only the visual appearance and is only related to diet as in the case of the salmonids.

The acceptability of a given food color may vary as we move from one population to another or from one social/economic group to another. While the emphasis of this chapter is on the chemical changes in natural pigmentation, the final acceptance of a product may depend on biases and habits of a market area.

Most pigment changes such as the thermal processing changes in chlorophyll, the bleaching of anthocyanins, the browning of fresh red

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TABLE 18.1. DISTRIBUTION OF NATURAL COLORING MATTER

Food	Heme	Carotenoids	Chlorophyll	Betalains	Anthocyanins	Flavonoids	Caramel	Melanins
Red meat	X							
Fish	X	X						X
Eggs		X						
Crustaceans		X						
Dairy products		X						
Green vegetables		X	X		X	X		
Root vegetables		X	X	X	X	X		
Fruits		X	X		X	X		
Cereal		X						
Syrups							X	

meat, or the off-coloring of flavonoids do not in themselves affect the quality of the product. They may reflect other changes and thus assume that of a primary characteristic. In the case of the destruction of carotene, the loss or change in color would result in poorer quality, but also may result in a loss of vitamin A activity.

Pigments degrade as a result of processing; however, these changes may be similar to those changes that occur through senescence. Thus, the object of both processing and storage is to slow down or stop these changes in pigments whether they are caused by chemical or biochemical reactions.

The synthetic carotenoids added to food will be discussed under the natural pigments. The FD & C pigments are much more stable than the natural pigments and thus discussion of their chemical changes is limited.

In this chapter the chemical changes on storage and processing of the following pigments will be covered: carotenoids, chlorophylls, heme pigments, anthocyanins, and betalains.

Clydesdale and Francis (1976) reviewed pigments from a general food science point of view, and Simpson *et al.* (1976) reviewed plant pigments primarily from a senescence perspective. The present review concentrates on the recent literature as it relates to chemical or biochemical changes in processing or storage.

The distribution of some natural coloring matters is shown in Table 18.1.

CAROTENOIDS

The carotenoid pigments are very widespread in fruits, vegetables, dairy products, eggs, and fish (Table 18.1). While animals are inca-

pable of a *de novo* synthesis, they can absorb the red and yellow carotenoids to color egg yolks, salmon flesh, chicken skin, bird feathers, etc. Certainly, a major contribution of these pigments to the diets of animals is the fact that a few of the 500 or so carotenoids can be split to form vitamin A. Thus, a loss of the color contributed by the carotenoid pigments results in a loss of quality as well as a potential loss of provitamin A. Figure 18.1 shows some representative carotenoids and sources. Those compounds with a half of the molecule resembling β -carotene have been shown to possess vitamin A activity. The trivial names (e.g., α -carotene) will be used in this chapter for the carotenoids rather than the IUPAC nomenclature (β , ϵ -carotene) (cf. Anon 1975). A convenient grouping of the carotenoids has been to separate them into hydrocarbons—carotenes and oxygen-containing compounds—xanthophylls.

The carotenoids are altered or partly destroyed by acids, usually but not always stable in bases, cleaved by some enzymes and sensitive to light exposure. Since these pigments are fat soluble, they are not subject to leaching losses. They are generally stable to the heat treatment involved in the canning operation, but are rapidly lost on dehydration due to oxidation. These reactions will be treated in more detail below.

Cis-Trans Isomerization

Generally, the most favored chemical state of the carotenoids is the all trans (Fig. 18.1). Mono-cis and poly-cis carotenoids do exist in fruits and vegetables. The tangerine tomato is characterized by poly-cis carotenes. The carotenoids are susceptible to cis-trans formation, particularly at elevated temperatures especially in the presence of light, I_2 , and acid. Lycopene could in theory exist in 1056 different forms, but because of steric hindrance probably only 72 forms exist (Moss and Weedon 1976). There are several consequences of the cis-trans isomerization reaction. It has long been known that the cis isomer has lowered vitamin A activity than the corresponding all trans (cf. Simpson 1983). Sweeney and Marsh (1973) fed rats cis-trans isomers of α - and β -carotene and found an enrichment of the all-trans structure in the feces which suggests that a geometric isomerization had taken place in the acid environment of the stomach. The insertion of a cis double bond in an otherwise all-trans conjugated system results in a loss of extinction and an absorption 2–5 nm shorter than the all-trans carotenoid.

The rate of the formation of cis isomers from the all trans is directly proportional to intensity of light at the main absorption bands, increase in temperature, and presence of catalysts, such as acid. Sin-

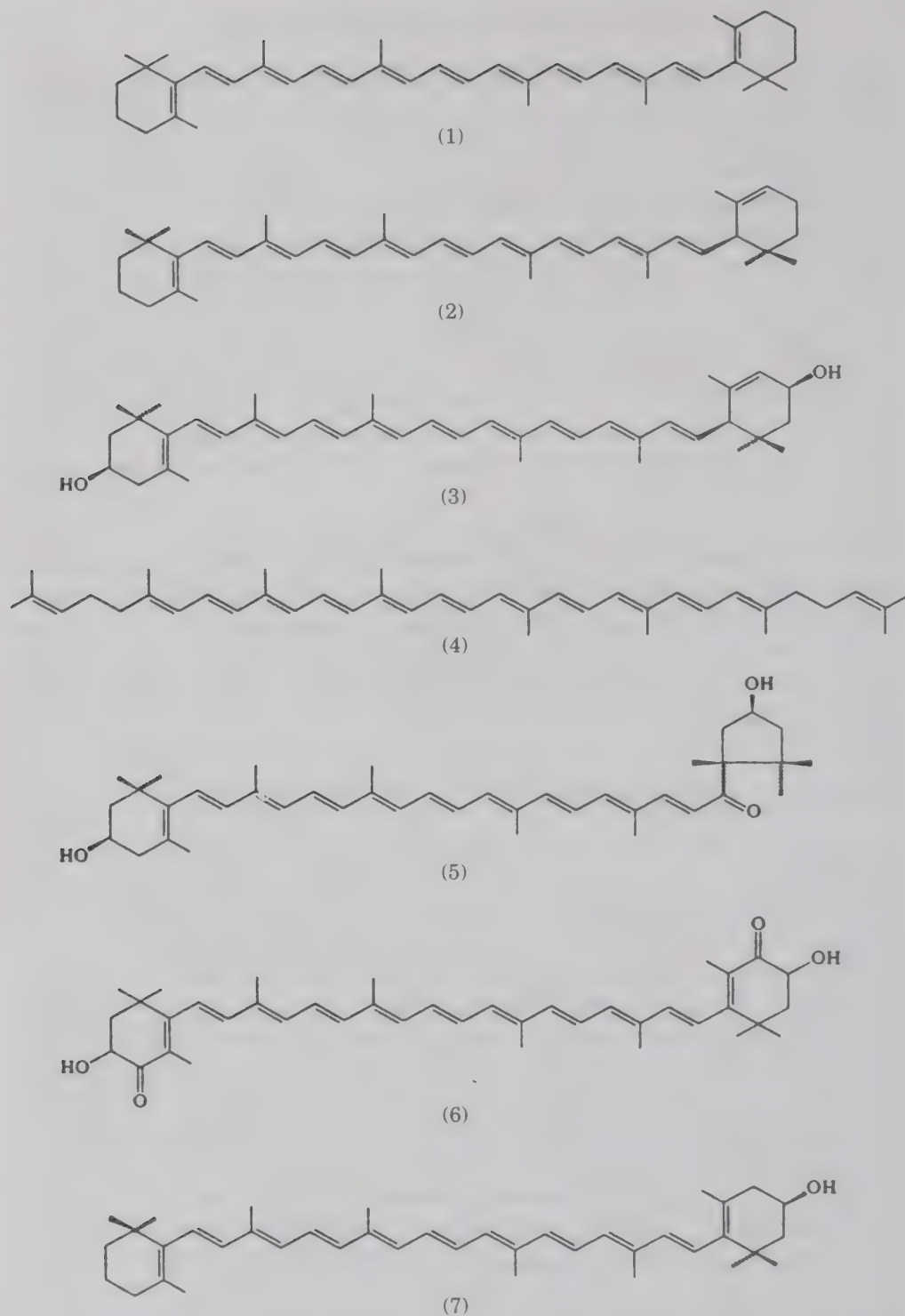


FIG. 18.1. Some representative food carotenoids and sources. (1) β -Carotene: green leaves, sweet potato, carrot; (2) α -carotene: carrot; (3) lutein: green leaves, corn; (4) lycopene: tomatoes, watermelon; (5) capaxanthin: pepper; (6) astaxanthin: fish, lobster; (7) β -cryptoxanthin: peaches.

gleton *et al.* (1961) showed that heat processing of pineapple produced enough acid to cause the formation of *cis* isomers. Thus, some isomerizations should occur in the processing of acid fruits which should result in lighter fruit.

The mechanism of the acid-catalyzed reaction probably involves the addition of a proton across the double bond with the formation of a carbonium ion on the second carbon. The reverse of this reaction should yield a *cis* or *trans* double bond. Cooking and extrusion at 150°C of a corn starch product resulted in the formation of a compound with the same spectral characteristics of 15-*cis*- β -carotene (Koné and Berset 1982).

Epoxidation

While it has been postulated that the initial breakdown product of the carotenoids occurs through the formation of epoxides, most of our

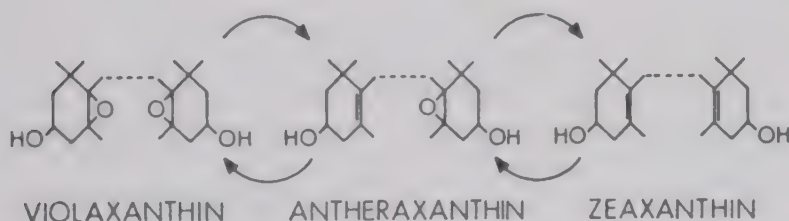


FIG. 18.2. Violaxanthin cycle pathway.

From Yamamoto (1979).

information has come from studies of biological systems. The violaxanthin cycle has been well studied in green plants during photosynthesis. Figure 18.2 shows the abbreviated structures of the violaxanthin cycle which is not an equilibrium since deepoxidation and epoxidation mechanisms differ. Both are enzymatic dark reactions, with the epoxidation reaction requiring reduced pyridine nucleotide and oxygen (Yamamoto 1979). The allenic monoepoxide, neoxanthin, is a common constituent of algae and higher plants.

Epoxides of β -carotene (Glover and Redfearn 1953; Goodwin 1958) and lutein 5,6-epoxide (Goodwin 1958) have been shown to be formed in leaves. Ben-Aziz *et al.* (1973) demonstrated the biochemical formation of acyclic and cyclic epoxides in tomatoes. Gross *et al.* (1971) reported the probable natural occurrence of mutatochrome, mutatoxanthin, and luteoxanthin in the juice of the Shamouti orange (Fig. 18.3). The seco-carotenoids, semi- β -carotenone and β -carotenone (Fig.

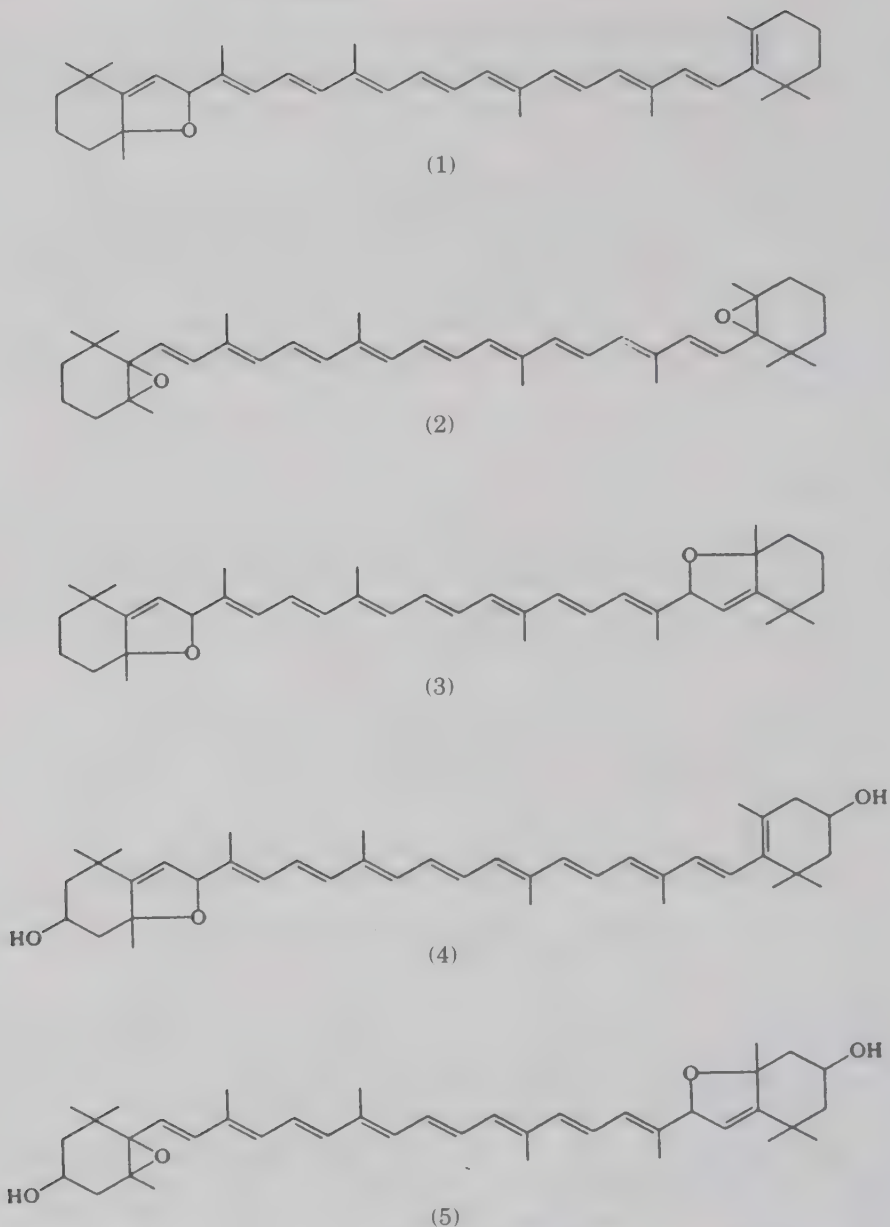


FIG. 18.3. Structures of some epoxide carotenoids. (1) Mutachrome; (2) β -carotene-5, 6, 5', 6'-diepoxide; (3) aurochrome; (4) mutatoxanthin; (5) luteoxanthin.

18.4), were isolated from all growth stages of *Triphasia trifolia* (Yokoyama and White 1970). While epoxides were not detected, it was suggested that the epoxide of β -carotene was the intermediate product.

A number of authors have worked with model systems and isolated

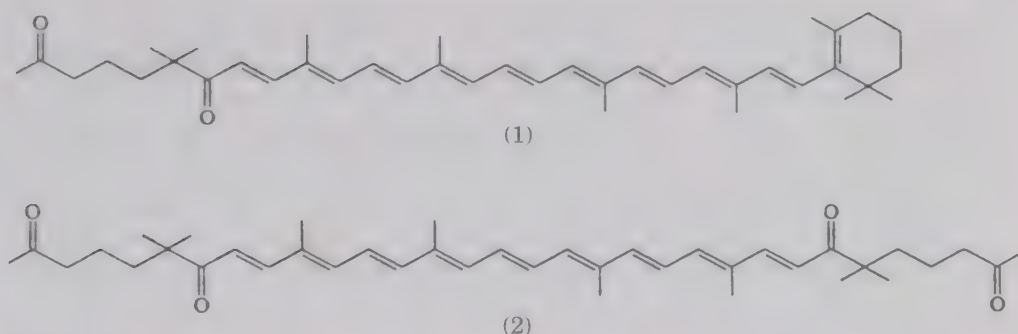


FIG. 18.4. Structures of some secocarotenoids. (1) semi- β -carotenone; (2) β -carotenone.

epoxycarotenoids. In other studies the bleaching of carotenoids was measured, and hence, the mechanism can only be inferred.

El-Tinay and Chichester (1970) studied the epoxide formation from β -carotene in a model system. The site of initial attack was the 5,6, 5',6' double bonds leading to 5,6 and 5,8 mono- and diepoxides. Diphenylamine, a free radical inhibitor, stopped the loss of β -carotene. Where the 4 and 4' position is substituted with a keto group (canthaxanthin), epoxidation occurs in the central chain, and alkali conditions rapidly split the molecule with the formation of carbonyl compounds (Cyronak *et al.* 1978).

Epoxide carotenoids have been isolated from bleached paprika under accelerated storage conditions (de la Mar and Francis 1969), the storage of canned orange juice (Curl and Bailey 1956), the processing of pineapple juice (Singleton *et al.* 1961), and the concentration of papaya puree (Chan *et al.* 1975) and tomato paste (Liu and Luh 1977).

Several epoxy carotenoids were isolated from stored, hydrogenated oils (McWeeny 1968). The complex nature of carotene destruction is shown by the isolation of *cis* isomers, epoxides, and long-chain carbonyl compounds from a coupled oxidation of β -carotene by a linoleate-lipoxygenase system (Friend 1958). Ramakrishnan and Francis (1979) isolated the products from a coupled oxidation of cryptoxanthin linoleate and showed the formation of epoxides and apocarotenoids.

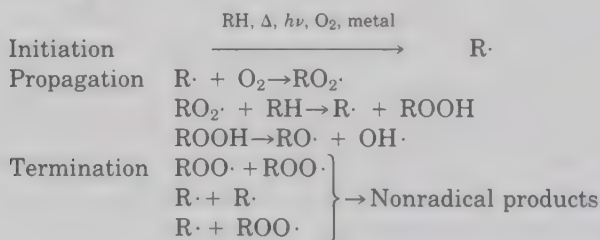
Oxidative Changes

The conjugated double-bond system makes the carotenoids especially susceptible to oxidative changes, usually leading to discoloration or bleaching. The reactions may be chemical or biochemical (i.e., enzymatic). The fresh tissue may contain carotene-destroying en-

zymes such as catalase, peroxidase, or lipoxygenase which are inactive in regard to the carotenoids being a substrate. The various operations involved in processing often "activate" the enzyme/substrate linkage and carotene loss ensues. Chemically activated oxidative changes are clearly indicated in heated oils, dried tissues, blanched and frozen vegetables, etc. While carotenoids are lost in senescent, processed, and stored foods, there is also a body of evidence documenting their role in protecting the functioning tissue against triplet sensitizers (e.g., chlorophyll), singlet oxygen, and free radicals (cf. Krinsky 1979). Recent epidemiological data, while not compelling, nevertheless suggests a role for carotene as a protective agent against cancer, probably through its antioxidant properties (cf. Peto *et al.* 1981).

Carotenoid oxidation in foods is generally associated with unsaturated fatty acids and is usually autocatalytic. The oxidation may or may not be enzymatic and is directly related to available water (A_w), O_2 , heat, and certain metals. Antioxidants were effective in showing the free radical nature of the reaction.

Karel (1980) in his review of lipid oxidation stressed the basic steps in the oxidation of lipids, including carotenoids. According to the lipid peroxide theory, lipids undergo an initiation step to produce free radicals which can be propagated and terminated. RH could be a carotene hydrocarbon (cf. Goldman *et al.* 1983).



Ouyang *et al.* (1980) isolated the decomposition products from a simulated deodorization of red palm oil and identified β -13- and β 14'-apocarotenals and β -13-apocarotenone. Figure 18.5 shows the position of the various chain splits. The isolation of an apocartenone indicates a secondary attack on the molecule. Ouyang *et al.* (1980) (Fig. 18.6) suggested a reaction pathway for the reaction starting with a propagation step with singlet oxygen and terminating with two apocarotenals. In practice red palm oil heated to 150°–210°C and held for 30 min results in almost a total loss of β -carotene (Mudambi and Rajagopal 1977).

Extrusion cooking destroys carotenoids (Koné and Berset 1982; Lee *et al.* 1978). Destruction in drying is a complicated process and is di-

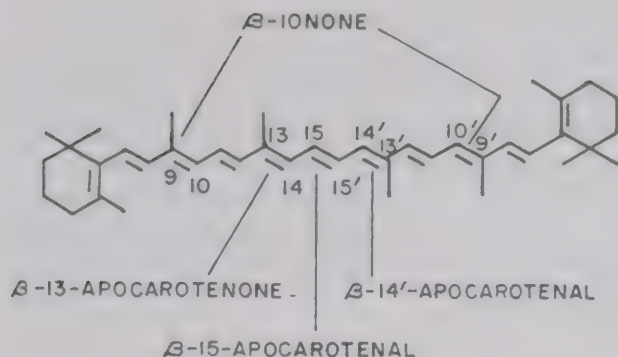


FIG. 18.5. Relationship between the attacking positions by oxygen formation of three carbonyl compounds.
From Ouyang *et al.* (1980).

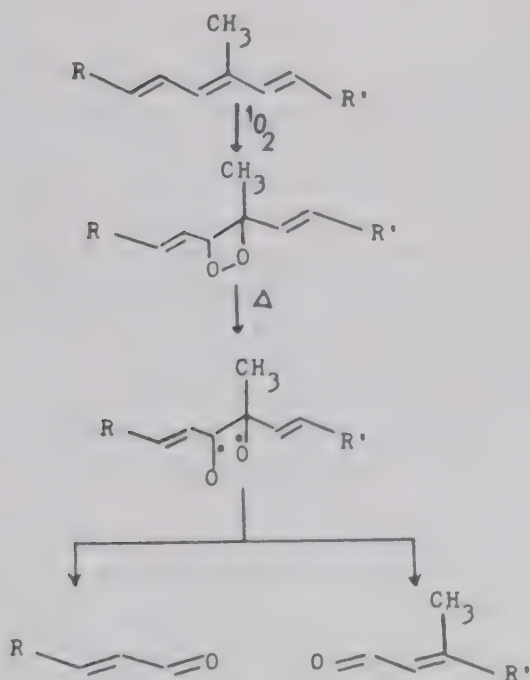


FIG. 18.6. Dioxetane mechanism for the oxidation of β -carotene.
From Ouyang *et al.* (1980).

rectly related to A_w . A number of authors report a protective effect during dehydration until a critical A_w is reached, at which point the further lowering of the A_w has a detrimental effect (Goldman *et al.* 1983; Karel 1980; Kanner *et al.* 1978; Kim and Rhee 1980). Karel (1980) discusses in some detail the effect of A_w on lipid oxidation. Kanner and Budowski (1978) showed that added ascorbic acid had little effect in the dry state, had a prooxidant activity at intermediate A_w , and an antioxidant activity at higher water concentrations. It is not surprising that even low levels of oxygen have a profound effect on the oxidation of β -carotene in dried foods (cf. Neto *et al.* 1981).

Enzymes have been shown to be the catalytic agents for carotene

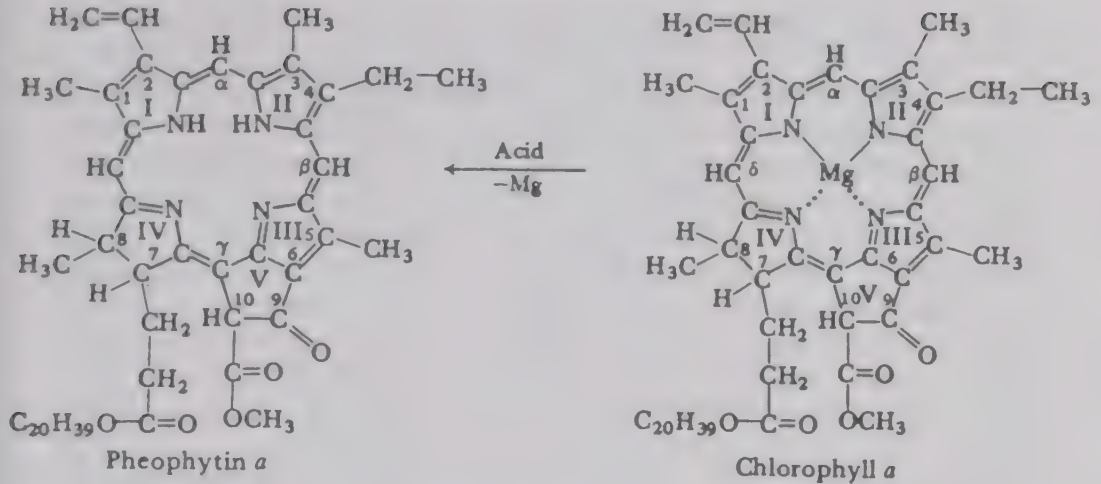
bleaching in a number of food and model systems. Red fish was shown by Tsukuda (1970) to be bleached by a lipooxygenase enzyme as astaxanthin, tunaxanthin, and β -carotene were degraded to colorless products. Myeloperoxidase from fish leukocytes was reported to cause the rapid discoloration of β -carotene in the presence of H_2O_2 and halide ions (Kanner and Kinsella, 1983). The major work on enzymatically catalyzed oxidation of carotenes has been with soya in which the carotenoids act as antioxidants or secondary substrates to the fat-oxidizing systems (Wever *et al.* 1974).

CHLOROPHYLL

Chlorophyll is the common name for a number of related tetrapyrroles which function in photosynthesis. The various derivatives are designated chlorophyll *a*, *b*, *c*, *d*, and bacteriochlorophyll *a* and *b*. Chlorophyll *b* is common in higher plants and in some algae. Chlorophyll *a* and *b* in a ratio of about 2:1 are usually encountered in foodstuffs. In the green plant the carotenoids protect chlorophyll from photodynamic destruction and thus all photosynthetic green plants contain chlorophyll and carotenoids (Krinsky 1979). Like the carotenoids, chlorophylls are easily degraded by similar chemical and biochemical reactions. A complete discussion of the structures, properties, and distribution of the chlorophylls has been published (Jackson 1976). For reviews, see Chichester and McFeeters (1971), Simpson *et al.* (1976), and Clydesdale and Francis (1976).

The nomenclature of chlorophyll is mainly based on a trivial naming system. Some of the important conversion products are as follows (cf. Clydesdale and Francis 1976):

1. Chlorophyll *a*: The structure of chlorophyll *a* is shown in Fig. 18.7. It is a magnesium-chelated tetrapyrrole structure with methyl substitutions at the 1, 3, 5, and 8 positions, vinyl at the 2, ethyl at the 4, propionate esterified with phytyl alcohol at the 7, keto at the 9, and carbomethoxy at the 10 position. The empirical formula is $C_{55}H_{72}O_5N_4Mg$.
2. Chlorophyll *a'* (*b'*): Probably a 10-epichlorophyll conversion caused by a weak alkali or heat.
3. Phytol: A 20-carbon alcohol with an isoprenoid structure.
4. Chlorophyll *b*: Chlorophyll *b* has the same configuration as chlorophyll *a* except that in the 3 position there is a formyl group rather than a methyl group. The empirical formula is $C_{55}H_{70}O_6N_4Mg$.

FIG. 18.7. Structure of chlorophyll *a* and pheophytin *a*.

5. Pheophytin *a*: Chlorophyll *a* minus magnesium.
6. Pheophytin *b*: Chlorophyll *b* minus magnesium.
7. Chlorophyllide *a*: Chlorophyll *a* minus phytol.
8. Chlorophyllide *b*: Chlorophyll *b* minus phytol.
9. Pheophorbide *a*: Chlorophyllide *a* minus magnesium.
10. Pheophorbide *b*: Chlorophyllide *b* minus magnesium.
11. Pyropheophytin *a* (or *b*): Pheophytin minus the carbomethoxy group (—CO₂CH₃) at C-10.

In general, the significant steps in chlorophyll degradation involve the loss of phytol to form chlorophyllide (or other related products, depending on where phytol is lost in the degradation pathway), loss of Mg²⁺ to form pheophytin, the loss of Mg²⁺ and phytol to form pheophorbide, and the loss of Mg²⁺ and the carbomethoxy group to form pyropheophytin *a*.

Heat Processing

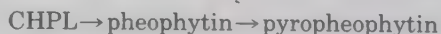
Even the most casual comparison of a fresh or frozen green vegetable with a canned product shows a drastic change in the chlorophyll pigment. While the label on the can may show a green color, the contents are brown due to chlorophyll destruction. A number of authors have reported E_a and D_{121} values for chlorophyll *a* and *b*, as well as for other pigments, thiamin, enzymes, *Clostridium botulinum* spores, etc. [cf. Table 9.2, Lund (1975)]. E_a is the amount of energy needed to place the molecule in a reactive state and D_{121} is the number of min-

utes to reach a 90% destruction of that molecule at the reference temperature.

The larger the E_a , the greater the rate change for a fixed increase in temperature. The E_a values reported by Lund (1975) would predict that a high-temperature short-time (HTST) treatment should improve the product as the values for chlorophyll are less than those for thiamin and much less than those for *C. botulinum* spores.

Clydesdale (1966), in fact, showed that HTST treatment of spinach puree resulted in a better retention of chlorophyll; however, what was gained in processing was lost on storage. After 3 months of storage, the color was similar for all process temperatures (Lin *et al.* 1971). These authors also reported that the HTST process resulted in a higher pH (favorable to chlorophyll retention), but that again on storage the pH of the HTST fell to approximately that of the 115.5°C process. There are a number of reports (cf. Clydesdale *et al.* 1972) of organic acids, including pyrrolidone-carboxylic acid (PCA), being formed on heat processing. Generally, the effect is less in HTST processing than in conventional processing, but on storage the HTST product produces more acid, hence a final result of no advantage to chlorophyll quality.

It has long been known that the Mg^{2+} is easily lost and replaced by two protons during heat processing or on storage in mild acid. The major reaction was thus the conversion of chlorophyll *a* to pheophytin *a* or *a'*. More recently, the resolving power of reverse-phase (RP) high-performance liquid chromatography (HPLC) has been applied to heat-processed foods. Schwartz *et al.* (1981) and Schwartz and von Elbe (1983) used RP HPLC to follow the degradation of chlorophyll *a* and *b* in fresh, blanched, and heated (121°C) spinach. Table 18.2 (Schwartz and von Elbe 1983A) shows that only chlorophyll *a* and *b* were isolated in the fresh and the blanched spinach, with no change in pH. On increased times at 121°C, the following degradation was observed with a decrease in pH and apparent first-order rate constants:



Activation energies E_a were reported for chlorophyll (CHPL) *a* and *b*, 25.2 and 22.5, and pheophytin *a* and *b*, 20.7 and 15.7 kcal/mol. Schwartz and von Elbe (1983A) examined several chlorophyll-containing products and also found the previously unreported pyropheophytin. The structure of pyropheophytin was confirmed to be pheophytin *a* or *b* less the carbomethoxy group at carbon 10.

There are a large number of papers on chlorophyll degradation in various foods and food products that have been reviewed (Simpson *et al.* 1976; Clydesdale and Francis 1976; Chichester and McFeeters 1971).

TABLE 18.2. CONCENTRATION OF CHLOROPHYLLS *a*, *b*, PHEOPHYTINS *a*, *b*, AND PYROPHEOPHYTINS *a*, *b* IN FRESH, BLANCHED, AND HEATED SPINACH PROCESSED AT 121°C FOR VARIOUS TIMES

	Chlorophyll		Pheophytin		Pyropheophytin		pH ^b
	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	
	(mg/g dry weight) ^a						
Fresh	6.98	2.49					7.06
Blanched	6.78	2.47					
Processed ^c							
2	5.72	2.46	1.36	0.13			6.90
4	4.59	2.21	2.20	0.29	0.12		6.77
7	2.81	1.75	3.12	0.57	0.35		6.60
15	0.59	0.89	3.32	0.78	0.09	0.27	6.32
30		0.24	2.45	0.66	1.74	0.57	6.00
60			1.01	0.32	3.62	1.24	5.85

From Schwartz and von Elbe (1983A).

^a Estimated error = +2%; each value represents the average of three determinations.

^b pH was measured after processing before pigment extraction.

^c Process times listed (minutes) were measured from the time the internal product temperature reached the retort processing temperature.

A number of attempts have been made to modify the pH and add Mg salts in a nonacid medium. While the color was retained, the texture and taste were degraded. Schanderl *et al.* (1965) observed the spontaneous regreening on storage of pureed, heat-processed green vegetables. The regreening was found to be a copper or zinc complex with pheophytin (cf. Jones *et al.* 1977).

Photodecomposition of Chlorophyll

Chlorophyll on exposure to light and oxygen is irreversibly bleached whether it is in a leaf (Goldwaite and Laetsch 1967) or in solution (Jen and MacKinney 1970A, B; Morris *et al.* 1973). In solution, red intermediates are formed which retain the nitrogen:phytol:Mg ratio of 4:1:1 (Morris *et al.* 1973). Whereas chlorophyll *a* is usually lost at a greater rate than chlorophyll *b*, no such difference was noted in photochemical reactions (Jen and MacKinney 1970A, B). During senescence most of the chlorophyll is lost, leaving the carotenoids and the water-soluble red pigments and phytol alcohol (Park *et al.* 1973).

Chlorophyll Stability in Dehydrated Foods

The level of A_w has a profound effect on the mechanism of chlorophyll destruction. At higher A_w , enzymatic action and microbial growth would be expected to take place. At low A_w , some protection seems to

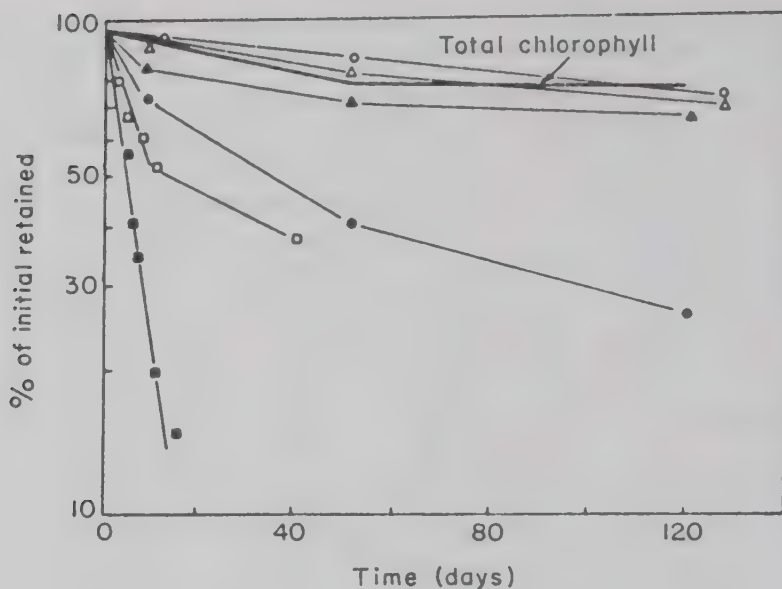


FIG. 18.8. Chlorophyll degradation in spinach puree.

A_w	Chlorophyll A
0	△
0.11	○
0.32	▲
0.52	●
0.62	□
0.75	■

From Lajolo *et al.* (1971).

be present due to compartmentalization, and water is not available for pheophytin formation.

Lajolo *et al.* (1971) studied chlorophyll degradation at A_w between 0.75 and 0 in spinach puree. Between A_w 0.32 and 0 the retention of chlorophyll was good when stored at 37°C. At A_w levels from 0.52 to 0.75 pheophytin formation was rapid (Fig. 18.8). Even at very low A_w values in the dark some pheophytin formation took place. Lajolo found that in common with heat processing, chlorophyll *a* degraded faster than chlorophyll *b*. A model system was constructed with a chlorophyll-cellulose complex. In contrast to the spinach system, all A_w levels resulted in high rates of pheophytin formation with additional oxidation. This observation indicates that the location in the cell protects the chlorophyll even in samples which had been frozen and dried.

High CO₂ Treatment

Short-term application of high CO₂ has been shown to be beneficial to the storage of many fruits and vegetables. However, in some fruits

and vegetables the injuries outweigh the benefits (cf. Wang 1979).

In the marketing of fresh broccoli, the florets turn yellow and the quality is thus reduced. Prolonged exposure to high CO₂ atmosphere limited the yellowing but produced off-flavor and off-odors (Wang and Hruschka 1977). Wang (1979) studied the effect of a limited high CO₂ treatment on the quality of stored broccoli. Broccoli was treated with 20, 30, and 40% CO₂ for 3 and 6 days and then stored in air at 5°C. The CO₂ delayed yellowing and retarded ethylene production and the loss of ascorbic acid and chlorophyll. The 40% CO₂ treatment for 6 days was rated not salable even though the florets remained green. The control was judged not acceptable. The effect of CO₂ on chlorophyll retention is probably related to its inhibition of ethylene generation and its action (Burg and Burg 1967).

Enzymic Changes

Chlorophyllase catalyzes the removal of the C-20 phytol side chain to form chlorophyllides. There are data to support a biosynthetic and a degradative role for this enzyme (Simpson *et al.* 1976). Recently, a procedure has been published for the purification of chlorophyllase by hydrophobic chromatography (Shimokawa 1982). Most of the activity of the purified enzyme was retained by heating at 55°C for 10 min. Total loss of activity was obtained by heating at 70°C for 10 min.

In the past there was much interest in chlorophyllase because of its stability to heating and because of the relatively greater stability of the chlorophyllides. However, Clydesdale and Francis (1976) state "unfortunately, in these studies it was not possible to produce enough chlorophyllide within the tissue to conclusively prove a gain in stability."

Green tissues that have not been blanched usually contain lipoxygenase-like enzymes. The enzymatic oxidation of chlorophyll has been reviewed by Simpson *et al.* (1976). The characteristics of the oxidation are similar to those discussed above under carotenoids. The most effective substrates are the long-chain polyunsaturated fatty acids, and chlorophyll appears to be bleached as a secondary substrate (Holden 1965). The reaction was inhibited by various antioxidants and by blanching.

HEME PIGMENTS

The color of red meat is due to two complex proteins—myoglobin and hemoglobin. In the animal, hemoglobin represents the major pigment; however, in well-bled meat, myoglobin is the major pigment.

While hemoglobin is four times larger than myoglobin, the oxygen capacity is the same as each has the same number of heme groups. Our interest thus is mainly in myoglobin, the efficient delivery of the iron from the food, and the color that it imparts to the food. The color of the heme portion depends on the state of the iron. Cured meat pigments will not be covered.

Recent reviews include Fox (1966), Clydesdale and Francis (1976), and Price and Schweigert (1971).

Chemistry

The structure of the isolated heme group is shown in Fig. 18.9. The iron in the tetrapyrrole is in a dynamic state between oxidation and oxygenation levels. Myoglobin (Fe^{2+}) in the presence of O_2 is reversibly converted to oxymyoglobin ($\text{Fe}^{2+}-\text{O}_2$). The former is bluish and the latter bright red. Metmyoglobin (Fe^{3+}) is the oxidized product which is characteristic of stale meat. In fresh meat under high oxygen pressure, the formation of oxymyoglobin is favored. Under lower oxygen pressure, the reduced pigment myoglobin is oxidized to metmyoglobin. An equilibrium exists as long as reducing substances are present. In older meat metmyoglobin predominates. Under an atmosphere of 1–2% carbon monoxide, carboxymyoglobin (MbCO) is formed. MbCO has a similar spectrum to oxymyoglobin and is somewhat more resistant to oxidation.

Packaging

In the modern supermarket all meat is sold in transparent wrappings. While other factors may be used, the consumer's first impres-

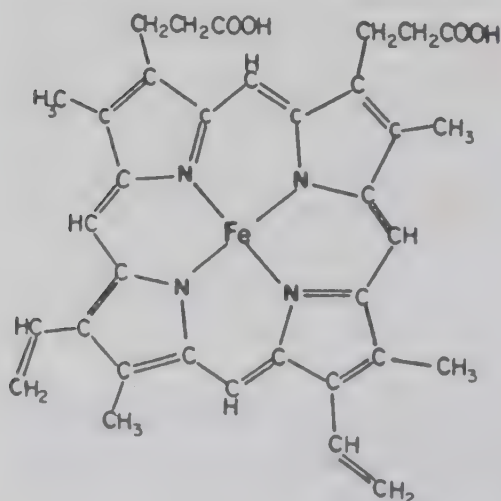


FIG. 18.9. Structure of heme.

sion of the quality of the meat is color. The formation of brown metmyoglobin in meat is usually in concert with the growth of spoilage bacteria. The package should then protect from bacteriological contamination and the formation of oxidized pigments.

George and Stratmann (1952A, B) reported that a high rate of oxidation to metmyoglobin occurred at partial pressures of oxygen in the range 1–20 mmHg. As the oxygen permeability of the wrapping decreases a point is reached where oxygen penetration is equal to oxygen utilization by the meat. This balance would favor oxidation. Landrock and Wallace (1955) found that the wrapping must have an oxygen penetration rate of 5 liters $O_2/m^2/day/atm$. Figure 18.10 details the conversions. The metabolic processes would cease and myoglobin would predominate where the film was an absolute barrier to O_2 . On removal of the film, oxymyoglobin would form and the meat would become red again.

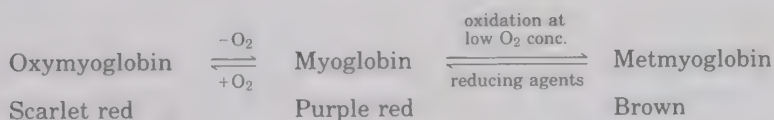


FIG. 18.10. Color cycle of fresh beef.
From Landrock and Wallace (1955).

Gee and Brown (1978) exposed ground beef patties to an atmosphere of 1% CO , 50% CO_2 , and 49% air under conditions where carboxymyoglobin would be formed. These authors found that the controlled atmosphere extended microbial shelf life by 4.5 days and maintained the bloom of fresh beef. The potential of CO toxicity should be a factor in its use.

Jurdi *et al.* (1980) studied the effect of combinations of CO_2 and N_2 atmosphere on deboned chicken meat. A 100% CO_2 atmosphere gave lower microbial plate counts but also resulted in a discoloration of the meat. The 30% CO_2 :70% air samples maintained a red color like the control. The formation of metmyoglobin was significantly higher in the 100% CO_2 high-fat deboned chicken meat than in the 30% CO_2 or 100% air treatments.

ANTHOCYANINS

The anthocyanins are a group of over 100 water-soluble plant pigments that are usually dissolved in the cell sap rather than in the lipoidal bodies. They greatly differ from the fat-soluble pigments in their stability to degradation and time of biosynthesis. It can be seen from Fig. 18.11 that as the strawberry matures, the levels of antho-

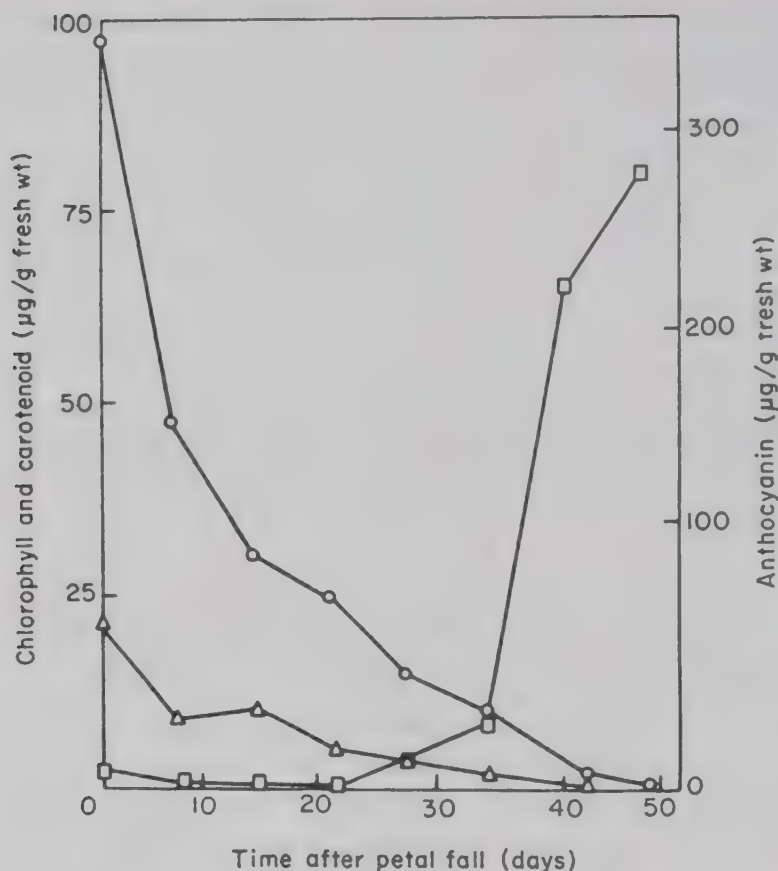


FIG. 18.11. Changes in pigment concentration per unit fresh weight in developing strawberry fruits. $\circ$, Chlorophyll; $\triangle$, carotenoid; $\square$, anthocyanin. From Woodward (1972).

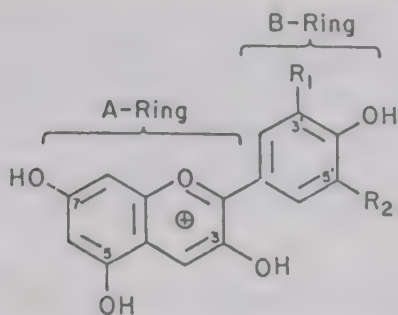
cyanins increase and the carotenoid and chlorophyll content becomes negligible. In the case of the autumn leaves the red anthocyanins are formed in senescent tissue.

There has been much interest in these pigments because of their importance to the color of fruits and vegetables, their sensitivity to color degradation through chemical and condensation reactions, and their potential use as natural pigmenters.

Jurd (1972), Singleton (1972), Simpson *et al.* (1976), Eskin (1979), and Clydesdale and Francis (1976) have reviewed the anthocyanin food pigments.

Substituent Effect on Stability

The general structure of the basic anthocyanidin cation structure is shown at the top of page 427.



The six aglycones (anthocyanidins) are shown in Fig. 18.12. It can be seen that the variations are in the substitutions on the B ring where R_1 and R_2 are H, OH, or OCH_3 . The aglycone rarely is found in food. These pigments usually occur as the glycosides (anthocyanin)

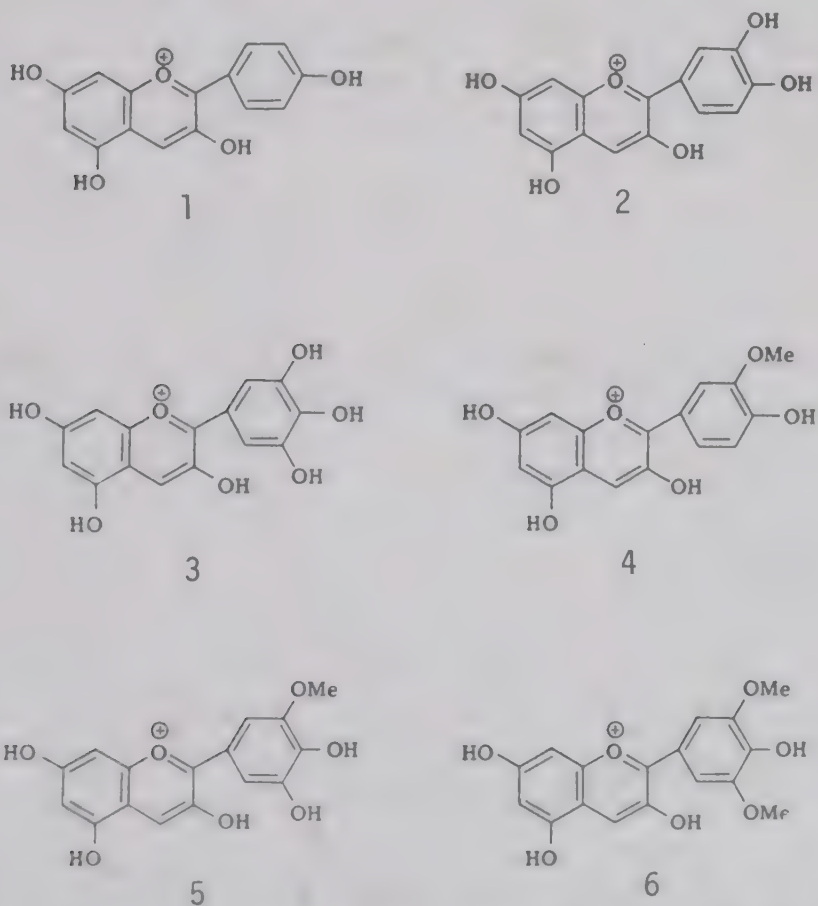


FIG. 18.12. Anthocyanidin structures: (1) pelargonidin; (2) cyanidin; (3) delphinidin; (4) peonidin; (5) petunidin; (6) malvidin.

of glucose, rhamnose, galactose, xylose, and arbinose. The glycosidic group at the 3 position imparts stability and the diglycoside is more stable to heat and light than the monoglycoside. A hydroxyl group at the 3' position increases the vulnerability of the compound to degradation (cf. Simpson *et al.* 1976). The anthocyanins may be acylated by a number of organic acids, including cinnamic acid.

Changes in Hue and Structure with pH Changes

These water-soluble pigments, especially the anthocyanidins, change hue in response to pH changes. They are most stable at low pH and are destroyed in the presence of oxygen at higher pH. Figure 18.13 shows the change in pH and in intensity of a buffered solution of cyanidin-3-rhamnoglucoside. At a pH of 0.71 the solution is red, while at 4.02 (and above) the pigment is almost colorless. The color returns to red on acidification. Harper (1968) suggested a pH transformation of pelargonidin (Fig. 18.14). The deep red flavylum ion (upper left) exists at pH 3. At a higher pH it is hydrated (central compound) to form the colorless pseudobase. This is in equilibrium with the keto

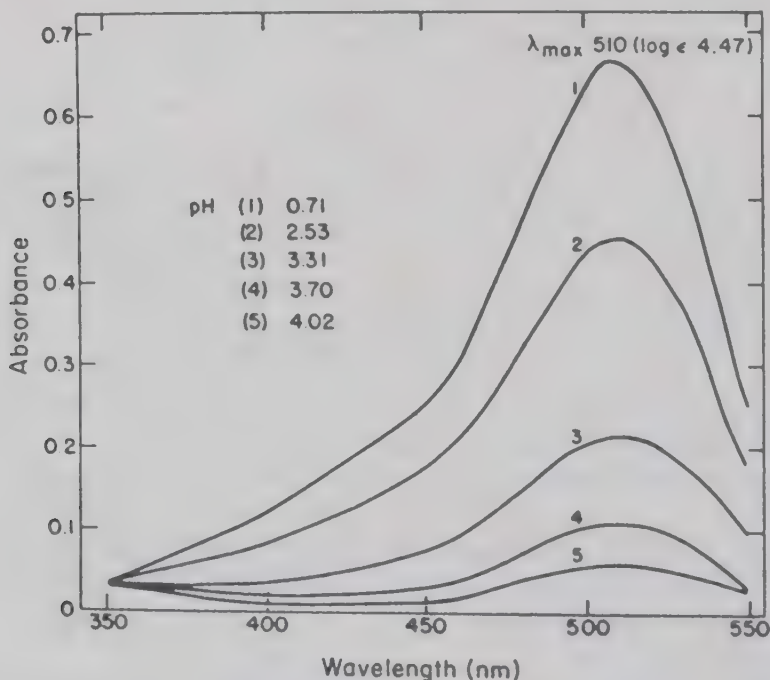


FIG. 18.13. Spectra of cyanidin 3-rhamnoglucoside (1.6×10^{-2} g/liter at equilibrium in buffered aqueous solutions.

From Jurd (1966).

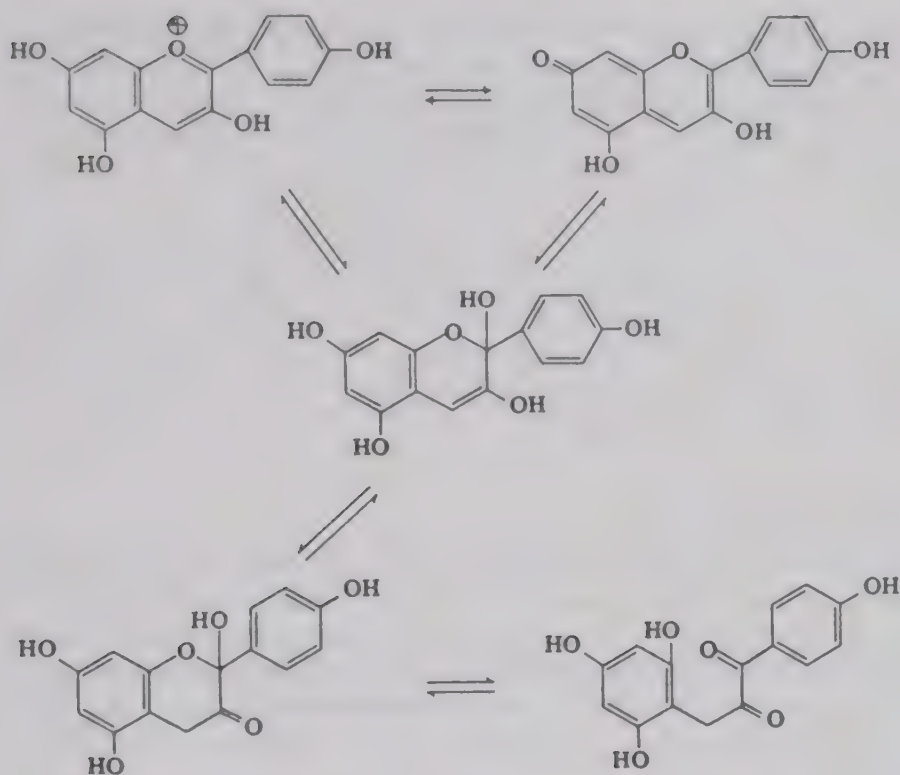
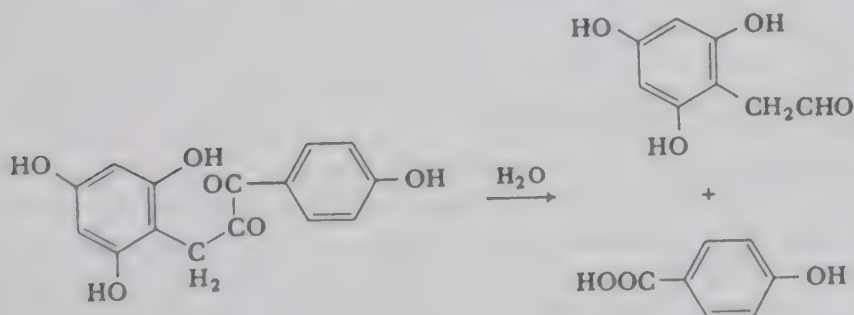


FIG. 18.14. Transformation of pelargonidin with pH.
From Harper (1968).

pseudobase which splits to form the α -diketone. The anhydrobase (upper right) forms at a pH of 6 and is purple. Cyanidin held at a pH 2–5 irreversibly splits (see below) to form phenolic benzoic acids.



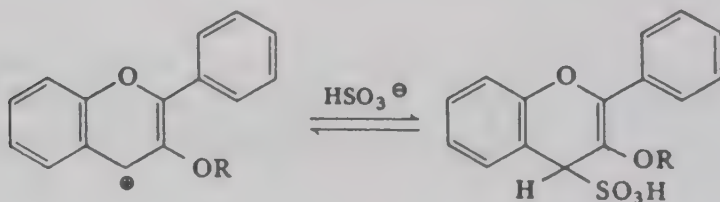
Wrolstad *et al.* (1970) studied the quality of several varieties of strawberries that had been frozen as the sliced and sugared product. The total and relative amounts of pelargonidin and cyanidin-3-glucosides, ascorbic acid, pH, total acidity soluble solids, and color deter-

minations were made. These authors found that only the pH measurement accurately predicted the color quality, with a pH of 3.51 or lower needed for good quality after freezing. Wrolstad *et al.* (1970) calculated that 37% of the pigment would be in the colored oxonium salt form at pH 3.21, while only 13% would be in the colored form at pH 3.81.

It had previously been shown by Lukton *et al.* (1956) that in the presence of oxygen, anthocyanin breakdown is dependent on pH and directly related to the level of pseudobase and inversely related to the level of the cation. While other factors contribute to poor quality of anthocyanin-containing fruits and vegetables, pH is a major variable in the stability of the color.

Sulfite Discoloration

Sulfur dioxide is used to prevent browning and to inhibit microbial growth in fruit. The treatment instantly bleaches the anthocyanins. The color, however, can be completely regenerated on acidification. Ribereau-Gayon (1959) suggested that the sulfur dioxide formed an addition product. Based on spectral and kinetic studies and synthetic compounds, Timberlake and Bridle (1968) suggested the following equilibrium:



Ascorbic Acid Degradation

Ascorbic acid, a natural nucleophile, has been shown in a number of reports to accelerate anthocyanin destruction in wines, fruits, and model systems (cf. Simpson *et al.* 1976). Hydrogen peroxide has been shown to cause the oxidation of flavylium salts to produce colorless enolic benzoates, benzoylestere, 3-substituted 2-phenyl-benzofurans or keto diols (Jurd 1972). Jurd (1966) proposed a mechanism for H_2O_2 oxidation of alkylflavylium salts (Fig. 18.15). Hrazdina (1970) proposed a mechanism for the H_2O_2 oxidation of malvidin-3,5-diglucoside. The extent of the autoxidation of ascorbic acid leading to hydrogen peroxide as the causative agent is still in some doubt. Jurd (1972)

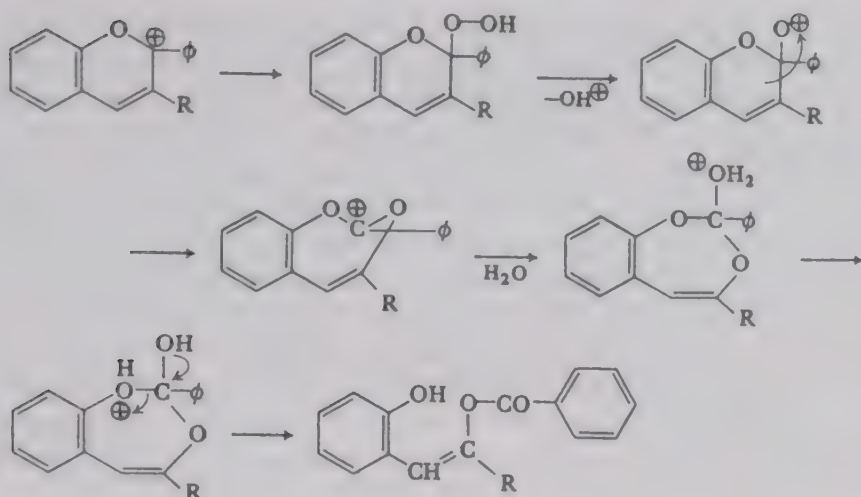


FIG. 18.15. Proposed mechanism for the H_2O_2 oxidation of alkylflavylium salts. From Jurd (1966).

raises the possibility of a condensation of the anthocyanin with ascorbic acid and other cellular constituents.

Metal Complexes with Anthocyanins

Anthocyanins that contain the *o*-dihydroxyl system (ring B) such as cyanidin and delphinidin glycosides chelate metals to produce a shift in hue from the nonchelated pigment. These complexes are more stable to pH effects and light than the parent compound. Cyanidin-3-glucoside forms a vivid red complex with aluminum at pH 5.5, and ferrous and ferric salts form a blue complex at a pH above 5.5 (Jurd 1972). Tin complexed with cyanidin and delphinidin glycosides is reported to cause the strong blue color in some berry juices (Pyysalo and Kerusi 1973). Canned peaches have been reported to take on a blue or purple discoloration due to a tin complex (Luh *et al.* 1962), and Chandler and Clegg (1970) suggested that the pink discoloration in pears was due to a cyanidin-tin complex.

Effect of Heat

The effect of heat on the stability of anthocyanin pigments during processing is well known (cf. Simpson *et al.* 1976; Jurd 1972). The kinetics are first order up to 110°C , but the pigments in several studies were degraded above 60°C . Spray drying of anthocyanin concentrates at outlet temperatures greater than 100°C caused extensive

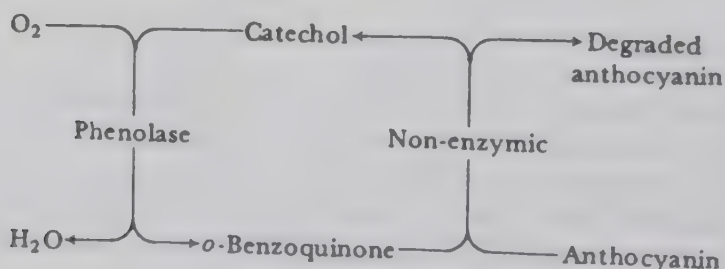
degradation of the pigments, whereas temperatures below 90°C resulted in minimal degradation (Main *et al.* 1978). Due to evaporation the actual temperature of drying will be below 90°C in spray drying and the use of a carrier tended to encapsulate the pigment. Calvi and Francis (1978) studied the stability of grape anthocyanins in various products. From other studies it had been shown that stability to thermal degradation increased as pH decreased. The exclusion of oxygen was important to anthocyanin stability. However, Calvi and Francis (1978) found a pH optimum of 3.2 with degradation resulting from a pH change in either direction and different results with the addition of various sugars. It would appear that the extrapolation from model system to the complex food is to be made carefully. The generally adverse effect of added sugar to thermal degradation of anthocyanins was shown by Daravingas and Cain (1968).

Simpson *et al.* (1976) discuss two possible mechanisms for the thermal degradation of anthocyanin: (1) the hydrolysis of the 3-glycosidic linkage to produce the more labile aglucone; and (2) hydrolytic opening of the pyrylium ring to form a substituted chalcone which degrades to a brown insoluble compound of a polyphenolic nature. Adams (1973) obtained support for both mechanisms from a model system and proposed a mechanism for the anaerobic thermal degradation of cyanidin glycoside resulting in phenolic compounds.

Effect of Enzymes

Enzymes capable of decolorizing anthocyanins or destabilizing the anthocyanins have been prepared from a number of sources (cf. Simpson *et al.* 1976). A glucosidase or anthocyanase has been shown to remove the stabilizing sugar which leads to the spontaneous decomposition of the aglucone. The second step has been discussed above.

A polyphenol oxidase that requires catechol for activation has also been described by a number of authors. Peng and Markakis (1963) proposed the following:



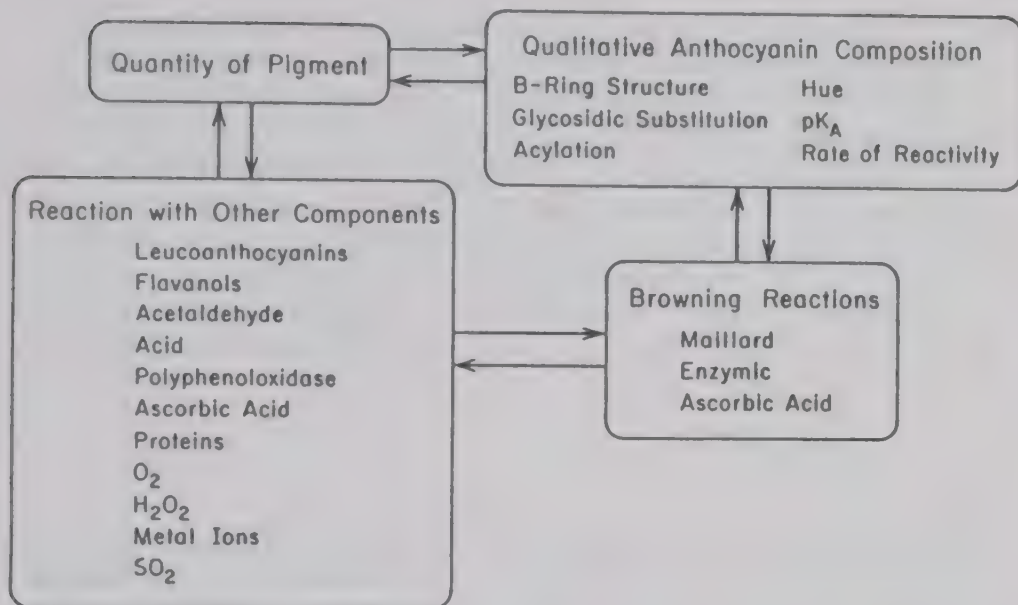


FIG. 18.16. Complexity diagram of color degradation in systems containing anthocyanin pigment.

From Wrolstad (1983).

Wrolstad *et al.* (1980) studied the effect of a microwave blanching procedure on a strawberry concentrate. The anthocyanin level was much higher at the beginning of storage in the blanched strawberry concentrate than the control, a fact which the authors attribute to enzyme inactivation. The blanched samples browned slower than the control. Enzymatic action was apparently not the cause of anthocyanin degradation in freeze-dried strawberry purees (Erlandson and Wrolstad 1972).

The preservation of the color of anthocyanin-containing foods is very complex. Loss of the bright red color may be due to loss of the color per se or to browning reactions which tend to dull the color of the food. Wrolstad (1983) shows the complexity of maintaining the color of anthocyanins (Fig. 18.16).

BETALAINS

The betalains are a group of water-soluble, nitrogen-containing vacuolar pigments which are separated on the basis of color into the red-violet betacyanins and the yellow betaxanthins. They are fairly restricted in their distribution (Piattelli 1976). A number of names have been used to describe these pigments and such terms as "nitro-

genous anthocyanins" have been particularly confusing. Recent epidemiological studies have incorrectly included the betalain-containing beet as a rich source of carotene (cf. Simpson 1983). While the foods containing the betalains are mainly restricted to the red beet *Beta vulgaris*, our interest in these pigments has increased due to the need for natural red pigment additives. Thus, the stability of the betalains has been the subject of several recent publications dealing with thermal degradation, photochemical bleaching, oxidation, and enzymatic changes. Recent reviews include Clydesdale and Francis (1976), Simpson *et al.* (1976), Piattelli (1976), and the significant papers by the group led by von Elbe.

Effect of pH

The normal pH value of the beet is approximately 5.5, and significant changes above or below this point are detrimental. Von Elbe *et al.* (1974) studied the color intensity of betanine solutions stored at various pH. No shifts in the absorption maxima occurred between pH 4.0 and 7.0; however, above 7.0 and below 4.0 some loss of extinction and a slight shift to longer wavelengths (pH 9.0) were noted.

Habib and Brown (1956) reported that beets processed between pH 3.5 and 5.5 retained the natural beet color. At 100°C some degradation occurred at pH 5.0, but the loss was rapid at pH 3 or 7 for either the pure pigment or beet juice (von Elbe *et al.* 1974).

Mild acid results in the hydrolysis of the glucose at carbon 5 to convert betanin or isobetanin (inversion at C-15) to the aglucones betanidin or isobetanidin (Mabry 1966) (Fig. 18.17).

Various food products were colored with beet pigments and stored at pH from 4.8 to 6.4, with less losses than comparably stored beet pigments. This result led von Elbe *et al.* (1974) to suggest a role for beet pigments as color additives.

Lashley and Wiley (1979) reported on the isolation of a betacyanine decolorizing enzyme from raw beet tissue. While the pH optimum was found to be at a pH of 3.4, the enzyme showed activity over a 2.0–4.8 range.

Effect of Heat

As was noted, even at pH of greatest stability, some losses are to be expected on heat processing (von Elbe 1974; Aurstad and Dahle 1973; Habib and Brown 1956). Von Elbe *et al.* (1981) found that 23% of the fresh beets were lost on blanching while 22% of the blanched

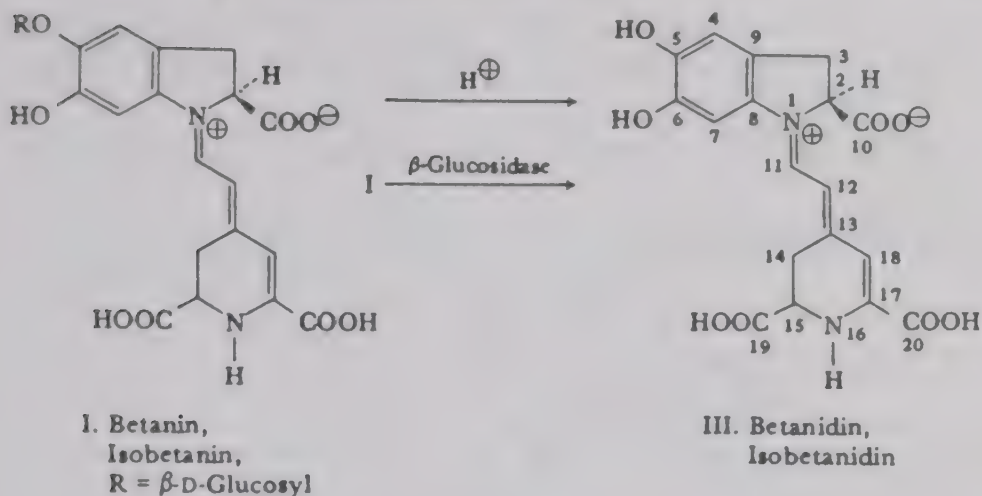


FIG. 18.17. Hydrolytic and degradative reactions given for several betacyanin pigments.

From Mabry (1966).

product was lost on canning at 126°C for 10 min. Thus, 41% was lost in the canning procedure. Increased heat treatment caused the formation of isobetanine from betanine. A partial regeneration of the beet color was noted at 10 hr.

Schwartz and von Elbe (1983B) identified some of the breakdown products of heated beet pigments and proposed a decomposition scheme (Fig. 18.18). The original betanine is proposed to degrade to cyclodopa-5-*O*-glycoside and betalamic acid on heating or in alkali. In acid or heat isobetanine is formed, and heat also is suggested to cause the decarboxylation of betanine.

The recombining of cyclodopa-5-*O*-glycoside and betalamic acid had been suggested as the cause of the regeneration after processing (von Elbe *et al.* 1981). Bilyk and Howard (1982) showed that isoascorbic acid when added before or after processing was effective in restoring the red color. These authors were able to show the appearance of betalamic acid during heating and a loss of betalamic acid and reappearance of betanine on the addition of isoascorbic acid. While betaxanthin was shown to degrade under these conditions, isoascorbic acid did not reverse the degradation reaction.

Oxidation

The detrimental effect of oxygen on beet pigments had been reported by Habib and Brown (1956). More recently, the degradation

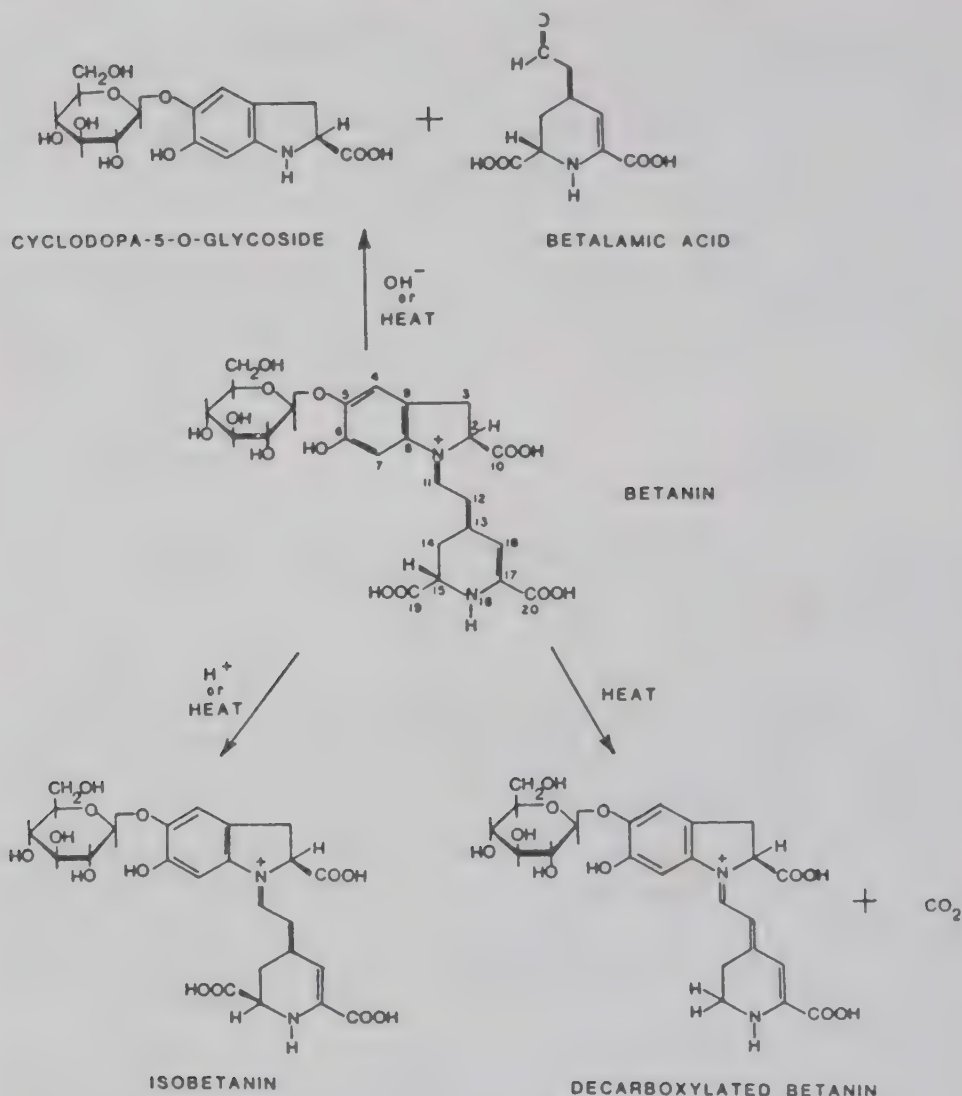


FIG. 18.18. Proposed decomposition products and reactions of betanin.
 From Schwartz and von Elbe (1983B).

kinetics of betanine oxidation was studied by Attoe and von Elbe (1982). These authors reported a pseudo-first-order rate in the presence of excess oxygen. The level of oxygen appears critical to the determination of the rate constant. Oxidation causes destruction of beet pigments in production of dry powders. Pasch and von Elbe (1975) reported that as the A_w dropped to 0.37, the stability of the betanine pigments increased. More recently, Saguy *et al.* (1984) showed the role of oxygen in the deterioration mechanism down to 0.5 A_w . Flu-

orescent light in the presence of oxygen was shown to be very effective in betanine destruction (Attoe and von Elbe 1981).

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Environmental Effects on Protein Quality

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INTRODUCTION

The environment of a food protein can exert profound changes on the functionality and nutritional quality of the protein. There are a number of degradative reactions resulting from the processing or storage environment which can cause undesirable changes in proteins. As a result of these reactions protein can exhibit losses in functionality, nutritional quality, increased risk of toxicity, and both desirable and undesirable flavor changes. Environment changes that can adversely affect proteins include heat in the presence and absence of carbohydrate, extremes in pH (particularly alkaline), and exposure to oxidative conditions, including those caused by light and those caused by oxidizing lipids. Nutrients are destroyed when foods are processed, largely because they are sensitive to pH of the solvent, to oxygen, light, heat, or combinations of these. The amino acid composition of food protein is of fundamental importance in determining nutritional quality and functionality. The literature referring to the influences of processing on proteins, particularly nutritional quality, can lead to two very different conclusions. First, one might interpret the literature to say that most commercial food processes have little or no effect on protein nutritional quality; on the other hand, one can find an

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equal body of evidence which shows that proteins are damaged even under the mildest of conditions. A number of factors must be considered when one attempts to resolve the significance of the issue. First, different proteins and food systems have very different susceptibilities to damage resulting from processing. One must also consider that in order to obtain measurable responses, experimental conditions are frequently much harsher than those to which a food might be exposed during commercial processing. Most commercial processes such as dehydration, canning, baking, and domestic cooking have only small effects on nutritional quality of proteins. There are, of course, the exceptions, for example, conditions where foods are exposed to very high pH, to extreme heat, or to peroxidizing lipids, any or all of which can produce significant changes.

In the past century our food delivery system has become much more complicated. There is now greater opportunity for various types of damage to occur to foods in processing, storage, distribution, and marketing, all prior to the greatest and most difficult variable to assess, final preparation in the home. It should also be pointed out that greater sophistication in processing by the food industry helps prevent or reduce the incidence of accidental "overtreatment" of a food.

The physical and chemical environments that a protein is exposed to during processing can result in a wide variety of changes in various amino acid side chains. Amino acids racemize and develop new cross-links in alkaline solutions. The results are losses in nutritional quality and significant changes in functionality. Arginine, cystine, threonine, serine, and cysteine are destroyed, whereas glutamine and asparagine are deaminated under alkaline conditions. In acid solutions, tryptophan is rapidly destroyed, particularly at elevated temperatures, and serine and threonine are slowly destroyed. Ultraviolet light destroys tryptophan, tyrosine, and phenylalanine. The sulfur amino acids are damaged by reaction products from lipid oxidation or by the addition of bleaching or oxidizing agents. All amino acids, especially lysine, threonine, and methionine, are sensitive to dry heat, browning, and radiations. Table 19.1 summarizes the susceptibilities of some of the amino acids to the types of damage that are reviewed in this chapter. The effects of heat, pyrolysis, browning, oxidation, photooxidation, and alkaline treatment on the nutritional quality, toxicity, and functionality of food proteins are reviewed.

INFLUENCE OF HEAT ON PROTEINS

Susceptibility to heat damage varies among different protein sources. Susceptibility is increased in the presence of various carbohydrates

TABLE 19.1. STABILITY OF AMINO ACIDS TO VARIOUS ENVIRONMENTS^a

	Light	Heat	Oxidizing lipids	Oxygen	Alkali	Acid
Lysine	S	U	U	S	S	S
Methionine	U	S	U	S?	S	S
Phenylalanine	U	S	S	S	S	S
Threonine	U	U	S	S	U	S
Tryptophan	U	S	U	S	S	U
Cystine/cysteine	U	S	U	U	U	S

^a S, Stable, no reported destruction; U, unstable, some destruction.

and other food constituents. Nutritional value of proteins can be significantly affected even when there is little or no apparent significant difference in amino acid composition. Even when pure proteins or foods containing minimum amounts of carbohydrate are heated, changes in nutritional quality can be observed. The thermally related changes in proteins can be broken into four basic categories as follows:

1. The first type of heat damage, which requires only mild heating, is an alteration in the tertiary structure of the protein and exerts no nutritional effect. These tertiary changes can have significant influence on functionality, for example, a loss in solubility. If the protein is an enzyme, it is likely, but not inevitable, that changes in tertiary structure will reduce or eliminate enzymatic activity. Thermal denaturation is of great significance in food technology because of the changes in the chemical and physical properties of the proteins. Globular proteins will exhibit changes (generally losses) in solubility, viscosity, osmotic properties, electrophoretic mobility, immunosensitivity, and chemical sensitivity. The changes are due to new reactive side chains of the protein being exposed as a result of the increased random coil being introduced in the protein. Fibrillar proteins when heated will suffer changes in elasticity, flexibility, and fibrillar length (Bender 1978).

Thermal changes will alter the properties of the food, improving or destroying the functional properties; for example, enzyme inhibitors such as trypsin inhibitors are denatured, avidin is denatured by heat, and egg albumin becomes insoluble, but in a useful form for consumption. Gluten is an example of a protein that loses its dough-forming properties as a result of too much heat. Most of these changes, however, have no measurable effect on the nutritional quality of the proteins themselves.

2. The second type of damage to protein associated with heat is the nonenzymatic browning or the Maillard reaction. This occurs primar-

ily between the ϵ -amino group of lysine and a carbohydrate. The lysine, after the very earliest stages of the reaction, becomes unavailable. Therefore, with the resulting Maillard reaction product bound to the protein, the solubility of the protein can change, and the color will change as the melanoidin pigments are formed.

3. The third type of change is the result of more severe heat treatment. Particularly lysine and cystine are sensitive to this type of thermal decomposition. Lysine and arginine side chains react with the free acids of glutamic and aspartic acids or with the amides to yield isopeptide cross-links which can impede digestion and exhibit major effects on functionality. Cystine is relatively sensitive and is converted to dimethyl sulfide as well as other products at temperatures of 115°C. Other amino acids can react with cystine and to a lesser extent methionine or histidine. A lactone ring is formed between a terminal carboxyl group and hydroxy amino acids (Bender 1978).

4. The fourth category of heat damage to protein is illustrated by examining the outside surface of roasted foods. The result of roasting is racemization of amino acid residues in the protein, or in the case of extensively heated material, complete destruction of the amino acids. Temperatures of 180°–300°C such as occur in roasting coffee, meat, and fish, and in the baking of some types of biscuits can result in some of the reactions mentioned above. These reactions also account for some of the flavor developed as a result of the roasting process.

One of the most easily observed thermal changes in protein is the change in conformation which affects solubility. Generally, protein solubility decreases with increases in the time and temperature of heat treatment, although there is considerable variation between proteins. Thermal denaturation of protein occurs when hydrogen and other noncovalent bonds, such as ionic and van der Waals bonds within the protein, are disrupted by the heating process. Thus, the normal secondary, tertiary, and quaternary structure of the protein is disrupted and the protein becomes "denatured." Damage to the protein proceeds from the native state to the predenatured, the denatured, and degraded, depending on the extent of thermal perturbation exerted by the environment. Figure 19.1 represents some of the stages that a protein might go through and some of the observable changes that would be expected. Interactions with several different functional groups (sulfhydryl, disulfide, tyrosyl) become more prevalent as the protein unfolds. These changes lead to a diverse and complicated series of reactions that ultimately lead to the precipitation of the protein. These

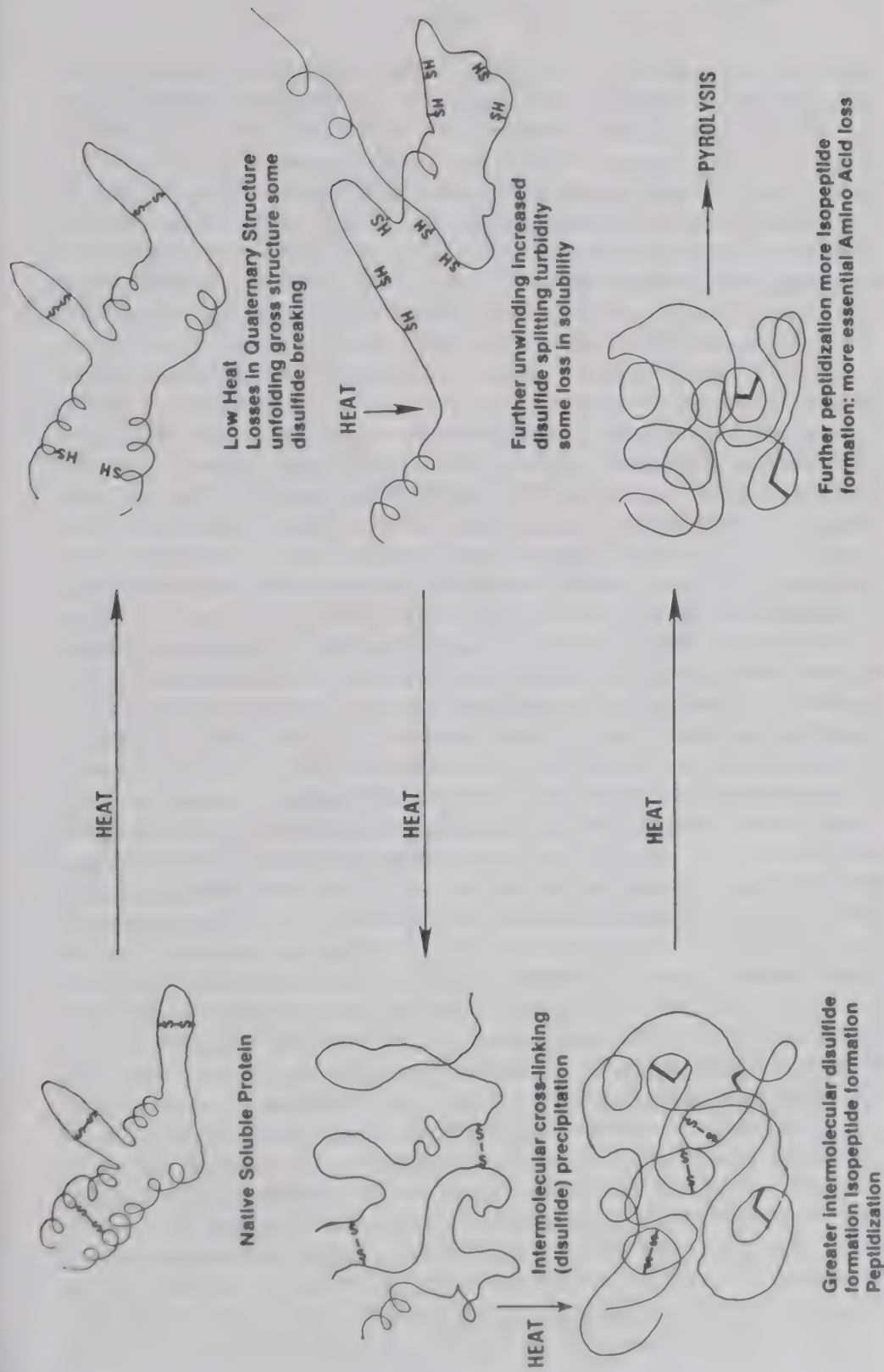


FIG. 19.1. Influence of increasing levels of isopeptide heat on structure of protein-S-S-disulfide bond.

reactions are generally irreversible. The interaction of water along with heat causes various ionic and polar groups in the protein to exert considerable influence on the folded conformations of the proteins (Edelhock and Osborne 1976). Moist heat frequently exerts more involved reactions in the protein than dry heat and is influenced greatly by pH and ionic strength. Mecham and Olcott (1947) demonstrated that dry proteins denature at different rates, but minimum solubility was reached for most proteins by 153°C. They observed marked losses in amino nitrogen and digestibility along with the loss in solubility. It was suggested that the formation of internal amides might account for some of these changes. Osner and Johnson (1974) studied the time/temperature relationships when dry, and 5% solutions of casein were heated. They established a clear relationship between total darkening and solubility of the protein. Significant losses were observed in aspartic acid, threonine, serine, lysine, and histidine. Schnack and Klostermeyer (1980) reported that when pure lactalbumin was heated, there was considerable desulfuration. Lactalbumin was most stable at pH 4.8 and became increasingly labile as the pH moved away from 4.8 either up or down. Lysozyme exhibited similar sensitivities.

Neucere and Cherry (1982) reported a study of the influence of moist and dry heat on the solubility of peanut protein. It was concluded that the effects of moist heat were more complex than the effects of dry heat. Proteins receiving dry heat exhibited a linear loss in solubility as a function of increasing temperature from 110° to 155°C. The wet-heated samples, however, showed a very different relationship. With the wet-heated samples the solubility plot over the same temperature range showed a sigmoidal curve with a minimum at 120°C. The solubility then decreased sharply after 145°C, the 155°C sample being nearly as insoluble as the dry-heated protein. In establishing protein functionality, it is apparent that effects of heat on solubility are of primary consideration. It should be kept in mind, however, that other properties such as emulsification capacity, whippability, gelation, texturization, extensibility, and water- and oil-binding capacity are also important functional characteristics of proteins in food systems.

Since food systems typically contain many different proteins, thermal processing can cause coaggregation among proteins in the mixture. Such coaggregations can be important in determining the characteristics of the food. The aggregations are also extremely difficult to study because they are chemically very stable (Neucere and Cherry 1982). When κ -casein is heated in the presence of β -lactoglobulin, a disulfide link is formed between the two proteins which reduces the thermal denaturation of the normally unstable β -lactoglobulin. The functionality of milk powder for baking is significantly enhanced by

heating the milk before drying to enhance the formation of the disulfide links between κ -casein and β -lactoglobulin (Kinsella 1982).

Oakes (1976) studied the thermal denaturation of bovine serum albumin by nuclear magnetic resonance. It was observed that intramolecular disulfide bonds, which are critical for maintaining native structure, are broken during thermal denaturation, and intermolecular disulfide bonds are formed. The amount of unfolding of the native protein upon thermal perturbation was less than predicted by other techniques. The model was described as folded or coiled coils in the native state. These larger coils appear to unfold on heating with moderate heat, but the helices remain unchanged by other techniques.

When proteins such as bovine serum albumin are heated, a lateral association of protein molecules occurs through a chain reaction of sulfhydryl groups with disulfides (Hospelhorn and Jensen 1954). Kolt-hoff and Tan (1965) found that heat denaturation of bovine serum albumin is not reversible unless the sulfhydryl groups of the protein are protected. The oxidation of cysteine to disulfide bonds in meat proteins occurs between 70° and 120°C. Further heating of meat can result in the sulfhydryls breaking down to yield hydrogen sulfide or further oxidation of cysteine to yield cysteic acid (Hamm 1977).

The reduction in the nutritional quality of heated proteins is attributed to the isopeptide cross-links formed as a result of the heat treatment. The decreased protein digestibility reduces the apparent bioavailability of most of the amino acid residues in the protein. Hurrell *et al.* (1976) described the formation of cross-links between the ϵ -amino group of lysine and the amide groups of asparagine or glutamine. Figure 19.2 shows the formation of these cross-links. It has been known for some time that heat induces a number of changes in the physical properties of proteins and that such changes can influence the digestibility and hence nutritional value of proteins (Mecham and Olcott 1947; Beuk *et al.* 1949). The nutritional value of heat-sterilized proteins was investigated by Carpenter (1960). Bjarnason and Carpenter (1969, 1970) demonstrated that simple acylation would not cause the observed loss in lysine availability. It was therefore speculated that isopeptide cross-links could be involved in the inhibition of the digestive process in heated proteins. Waible and Carpenter (1972) investigated the utilization of free isopeptides as a source of lysine. They demonstrated that chicks could use the glutamyllysine as a source of lysine. Otterburn (1983) reported that homogenates of the small intestine showed considerable activity toward the isopeptides. Raczynski *et al.* (1975) reported that glutamyllysine will pass the gut wall in model systems. One might speculate that although the isopeptides are absorbed and hydrolyzed in the gut wall, their presence in protein

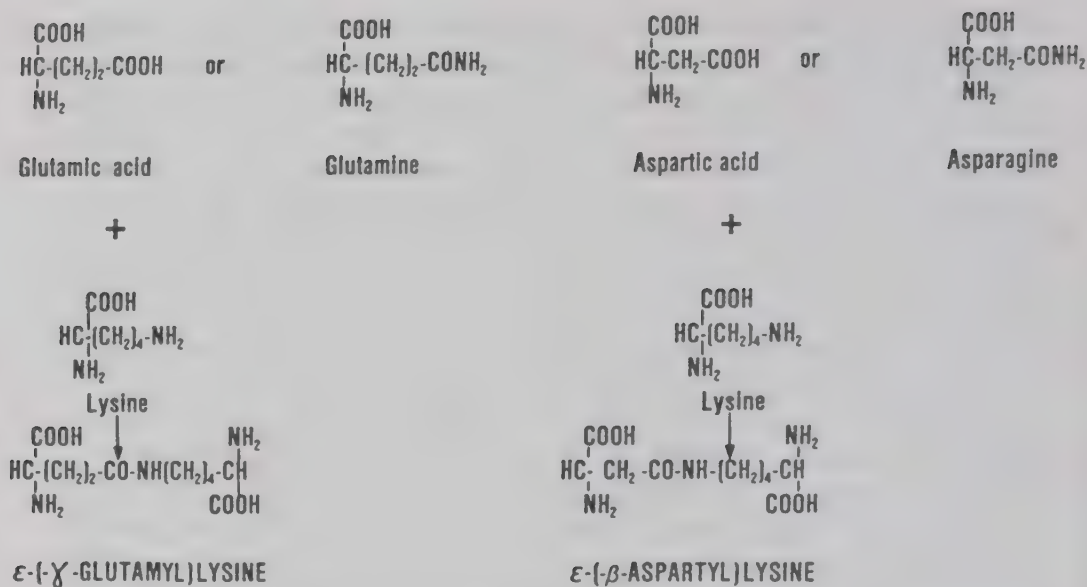


FIG. 19.2. Proposed reaction scheme for the formation of ϵ - ω isopeptides in heated proteins.

may slow the overall digestion, particularly if one considers that lysinoalanine, another cross-link to be discussed later, could also be present in severely heated proteins.

Limited research has been carried out on the nutritional significance of severely heated proteins which are known to contain isopeptide cross-links. One such was conducted by Hurrell *et al.* (1976) using severely heated chicken muscle protein fed to rats. It was found that although the digestibility of the protein was greatly reduced, the digestibility of the isopeptides was at least as good as the protein. One possible explanation is that the isopeptides are not absorbed in the ileum, but pass to the colon where they are hydrolyzed by the microflora (Otterburn 1977). Thus, there is evidence that suggests that glutamyl- and aspartyllysine are found in severely heated proteins, and the levels of isopeptides seem to correlate with the intensity of heat treatment (Otterburn 1983). Table 19.2 shows the concentrations of isopeptides in proteins having received various heat treatments. From the data it can be seen that as the intensity of heating increases, the level of isopeptides also increases. In addition to increasing isopeptide formation the severity of damage to the remainder of the protein increases with increasingly intense heat treatment.

The thermal decomposition of several amino acids has been studied in detail by several workers (Simmonds *et al.* 1972; Ratcliff *et al.* 1974,

TABLE 19.2. ISOPEPTIDES IN HEATED PROTEINS

Protein	Amino acids (g/16 g N)			
	Time of heating (hr)	Temp (°C)	Aspartyl-lysine	Glutamyl-lysine
Bovine hemoglobin	27	121	0.75	0.19
Blood meal	Low temp.		0.33	0.32
Blood meal	High temp.		0.25	0.44
Egg albumin	57	115	0.27	0.12
Zein	57	115	0.11	0
Lactalbumin	27	85	0.04	0.06
Lactalbumin	57	115	1.04	0.74
Lysozyme	27	121	0.43	0
Casein	27	115	0.14	0.48
Chicken muscle	4	121	0.30	0.20
Chicken muscle	8	121	0.40	0.40
Chicken muscle	27	121	0.80	0.90

From Otterburn (1983).

Lien and Nawar, 1974A, B; Breitbart and Nawar, 1979). In Table 19.3 are summarized the decomposition products from some of these severely heated amino acids. Uchiyama and Uchiyama (1981) reported that free radicals are formed in protein or lysine heated at 200°C for 22 min. It is of concern that these free radicals appear to be stable in water and in digestive juices. It is likely that similar reactions take place when proteins reach equivalent temperatures, although protein reactions will be complicated by the presence of the peptide bond, oxygen, and other components in the food.

An aspect of thermal decomposition of foods that must be considered is the possible formation of toxic products. Sugimura and Nagao (1979) found more mutagenic activity on the surface of flame-broiled fish and beef than could be accounted for by the benzo[a]pyrene which comes from pyrolyzed fat. This observation led to the discovery of several mutagens which are of protein or amino acid origin. Two of the most toxic mutagens are derived from tryptophan. In Fig. 19.3 are shown the structures of two of the products, Trp-P-1 and Trp-P-2. Trp-P-1 is less mutagenic in the Ames test than Trp-P-2, but Trp-P-1 is much more potent carcinogen (Sugimura *et al.* 1977; Ishikawa *et al.* 1979; Takayama *et al.* 1979). It is important to note that these compounds are formed only at temperatures in excess of 300°C.

The research reviewed in this section summarizes some of the work which has been done concerning the effects of heat on proteins. It is difficult to separate the influence of heat alone from the combined effects of heat in the presence of carbohydrate and oxidizing lipid. In

TABLE 19.3. THERMAL DECOMPOSITION PRODUCTS OF VARIOUS AMINO ACIDS^a

Lysine	Alanine	Valine	Leucine	Isoleucine
Piperidine	Ammonia	Ammonia	Ammonia	Ammonia
Tetrahydropyridine	Carbon dioxide	Carbon dioxide	Carbon dioxide	Carbon dioxide
Pyridine	Carbon monoxide	Carbon monoxide	Carbon monoxide	Carbon monoxide
2-Methylpyridine	Ethane	Propane	Isobutane	Butane
Pyrrole	Ethene	Propene	Isobutylene	Butene
2-Methyl-6-ethylpyridine	Propene	Isobutane	Isopentane	Isobutane
N-Acetylpyrrolidine	2-Butene	Isobutylene	3-Methyl-1-butene	2-Methyl-1-butene
2-Aminopyrrolidone	Acetaldehyde	Acetone	Acetone	2-Butanone
2-Piperidone	Ethylamine	Isobutyraldehyde	Isobutyraldehyde	methylobutyraldehyde
2-Oxohexamethyleneimine	N-Ethylidene-ethylamine	Isobutylamine	Isovaleraldehyde	2-Methylbutylamine
2-Methylpiperidine	2-Methyl-5-ethylpyridine	N-Isobutylidene-isobutylamine	Isobutylamine	N-(2-Methylbutylidene)-2-methylbutylamine
Hexamethyleneimine	N-Ethylpropionamide	Diisobutylamine	Isoamylamine	Bis(2-methylbutyl)amine
N-Methyl-N-propylisopropenylamine	Propionamide		N-Isobutylidene-isoamylamine	
Cyclohexanone			N-Isoamylidene-isoamylamine	
2-Methylpyrrole			Diisoamylamine	
2,5-Dimethylpyridine				
2,4-Dimethylpyridine				
N-Ethylacetamide				
2-Propylpyridine				
3-Ethylpyridine				

From Lien and Nawar (1974A, B) and Breitbart and Nawar (1979).

^a Heat treatments were from 220° to 250°C for 1 hr at 10^{-3} Torr vacuum.

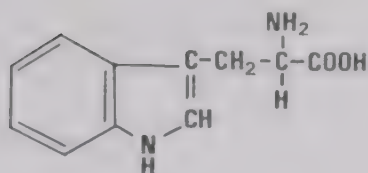
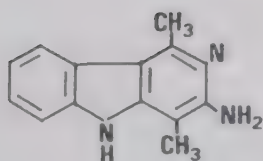
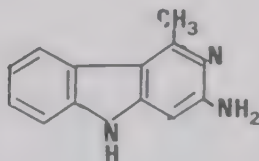
**Tryptophan****Trp-P-1****Trp-P-2**

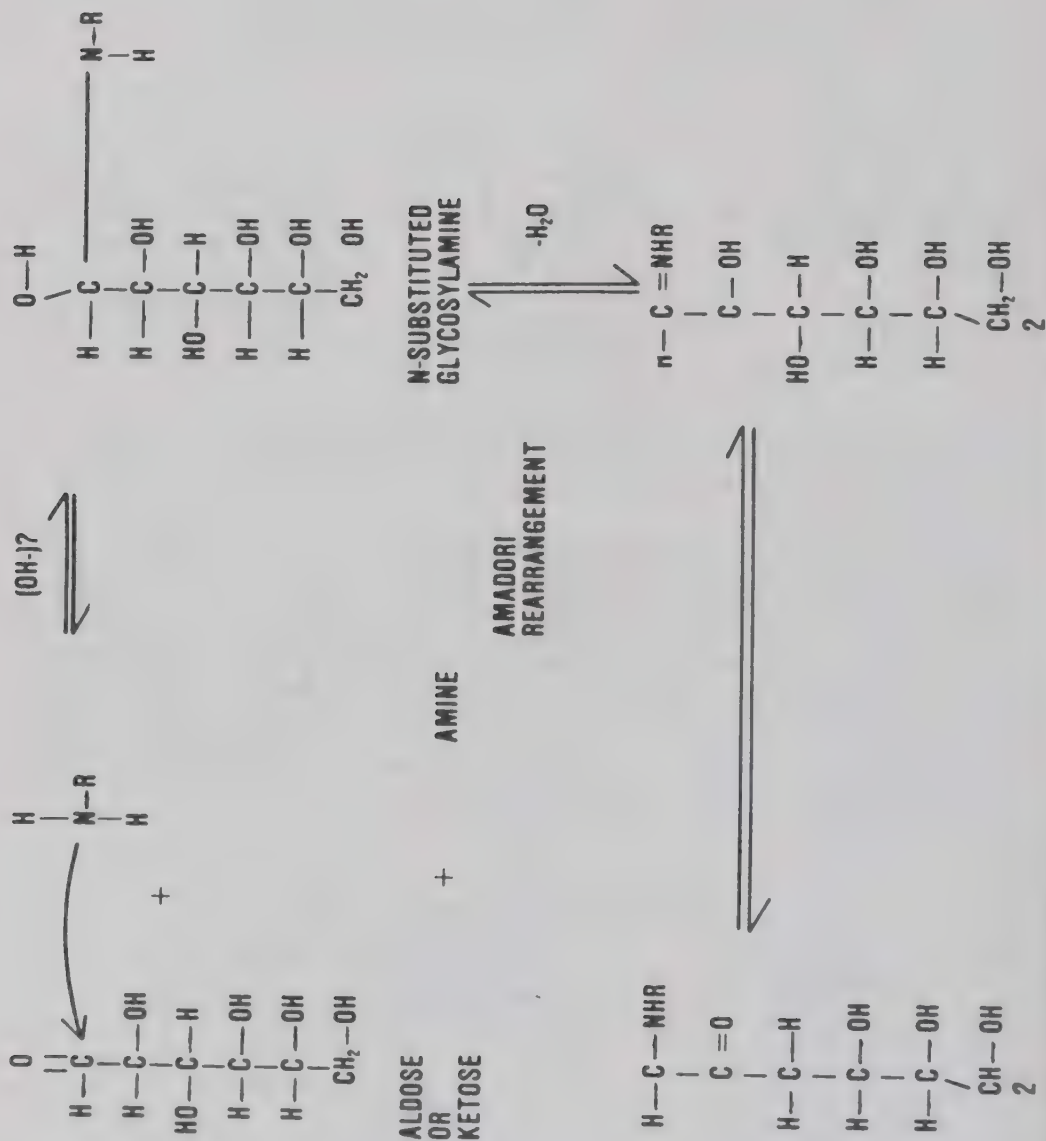
FIG. 19.3. Structures of tryptophan, Trp-P-1 (3-amino-1,4-dimethyl-5H-pyridol[4,3-*b*]indole, and Trp-P-2 (3-amino-1-methyl-5H-pyrido[4,3-*b*]indole).

Table 19.4 are summarized the types of degradations one might expect to see in proteins at various temperatures when proteins are heated alone.

Pieniazek *et al.* (1975) studied the effects of heat on casein. It was suggested that when high temperatures are applied to proteins the sulfur amino acids may be oxidized to unavailable forms. In both in vivo and in vitro studies of heated casein and heated casein plus glucose, tryptophan and tyrosine were lost and the sulfur amino acids were oxidized to nonavailable forms. It was also observed in this work that the presence of cysteic acid in a peptide reduced the digestibility of the polypeptide in the region of the cysteic acid.

TABLE 19.4. INFLUENCE OF HEAT ON PROTEINS

Temperature	Change or degradation
70°–80°C	Disulfide bond splitting Opening of helical structures Precipitation of very sensitive proteins
80°–100°C	Further unraveling of protein structure Losses in disulfides
100°–150°C	Lysine decomposition Serine and threonine losses Isopeptide formation Lysinoalanine formation
200°–250°C	Pyrolysis of all amino acids
>300°C	Formation of carcinogenic pyrolysis products



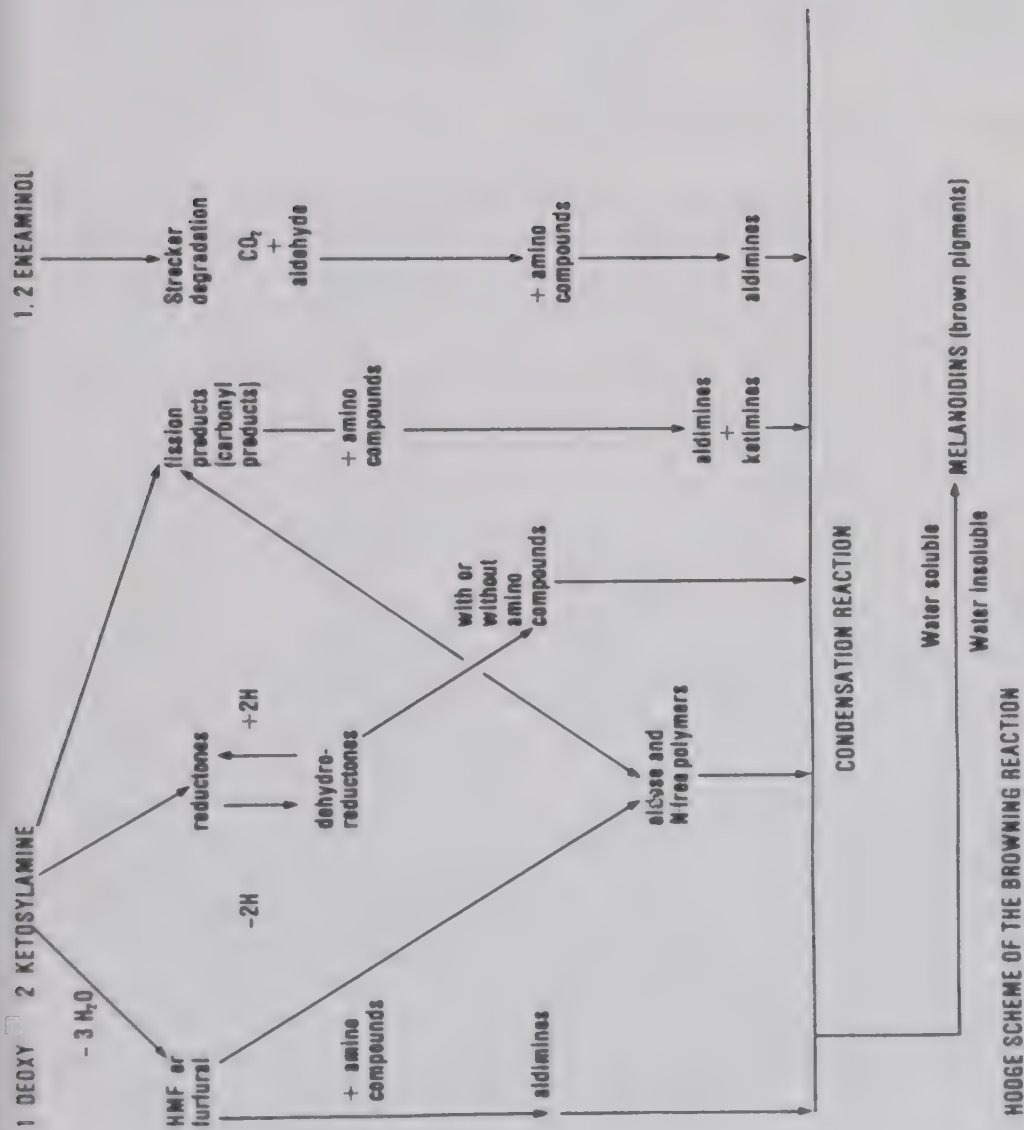


FIG. 19.4. Typical browning reaction pathways.
From Hodge (1953).

It should be pointed out again that many of these reactions do not reflect conditions reached in normal food processing. It is particularly important to recognize that in processes where proteins reach high temperatures it is usually only on the surface. It is also important to realize that many of the pyrolysis products are important to the development of flavor in baked and roasted foods.

NONENZYMATIC BROWNING

The browning or Maillard reaction is without a doubt the reaction most of us think of first when considering damage to proteins during food processing. Of the reactions discussed in this chapter, the browning reaction has the most significance from the nutritional point of view. The denaturation of protein may be more significant in terms of effect on protein functionality. The browning reaction has been the subject of considerable discussion (Adrian, 1974; Waller and Feather, 1983), including Chapter 13 of this volume. The basic chemistry of the browning reaction was described by Hodge (1953) and later by Reynolds (1969). Figure 19.4 summarizes the principal stages of the browning reaction. It is important to keep in mind that while the browning reaction can account for substantial losses in nutritional quality of proteins, it is also critical to the development of flavor in foods. Generally, foods are browned enough to substantially influence the nutritional quality would seem overcooked and of less than desirable flavor. The environment of the protein or food can have a substantial effect on the nature and extent of the observed browning.

The Maillard reaction occurs during both storage and heat treatment. The reaction is slow at room temperature and increases with temperature (Maillard 1912; Lea and Hannan 1949; Overby *et al.* 1959). According to Adrian and Favier (1961), equivalent losses in amino acid would be observed when heating a lysine glucose solution for 25 hr at 120°C or for 6 hr at 130°C. On a more practical level, Mauron (1964) determined the total and available lysine in milk dried in various ways. Results of that work are shown in Table 19.5. Although the chemical and biological methods differ slightly in the absolute answers obtained, the trends clearly show the loss of lysine with increasing intensity of heat treatment. The loss of the essential amino acid lysine serves as the best single indicator of damage to the protein from the browning reaction.

Another critical environmental effect on the browning reaction in protein is the influence of pH. Acidification inhibits the browning reaction, and raising the pH above 7.0 greatly enhances browning. The

TABLE 19.5. TOTAL AND AVAILABLE LYSINE IN COMMERCIAL MILKS

	Lysine (g/16 g N)			
	Spray powder	Evaporated	Roller dried	Overheated roller powder
Total lysine	8.00	7.60	6.80	6.10
Available lysine				
In the rat	8.10	6.10	4.00	2.00
Chemical assay	8.20	6.20	4.50	2.30

From Mauron (1964).

TABLE 19.6. PER OF BREAD AND TOAST

Bread in diet	PER
Casein (control)	2.50
Whole bread	1.02
Crust	0.42
Crumb	1.47
Crumb (higher baking temp.)	0.90
Light toast	0.64
Medium toast	0.45
Dark toast	0.32

From Tsen *et al.* (1983).

Maillard reaction increases approximately linearly from pH 3 to 8.0 (Lea and Hannan 1949; Adrian 1963; Lea 1950). This is also the region where most foods are subject to heat treatment.

The browning of bread during the baking process is essential to the development of what we consider to be bread flavor. Lysine is the first limiting amino acid in wheat and therefore in bread, and this limiting factor can be aggravated by the baking process (Jansen and Ehle 1965; Jansen *et al.* 1964; Tsen *et al.* 1983). Tsen *et al.* (1983) have reported the protein efficiency ratio (PER) of bread, bread crumb, and bread toasted to various levels. Their results are summarized in Table 19.6. These data represent breads baked at different temperatures, but clearly illustrate the loss in nutritive value as a result of intense heating. In the same study Tsen *et al.* (1983) reported the influences of high-temperature short-time heating of pizza doughs on the amino acid profiles of the pizza. The results are shown in Table 19.7. Lysine, and to a lesser extent cystine, tyrosine, and threonine are lost in the crust after baking. The losses ranged from 7.1% in whole-wheat pizza crust to 19.4% in commercial pizza crust. It was proposed that the losses in nutritive value of pizza crust could be correlated with losses in lysine.

A major environmental factor which influences the extent of brown-

TABLE 19.7. AMINO ACIDS CONTENTS IN WHEAT FLOURS AND PIZZA CRUSTS^a

Amino acid	WF ^b	WWF	PCW	PCWB	PCWW	PCWWB	PCC	PCCB	PCCM	PCCMB
Aspartic acid	4.30	5.39	4.19	4.12	5.33	5.36	4.72	4.49	4.79	4.69
Threonine	2.56	2.89	2.66	2.65	2.98	2.91	2.75	2.74	2.94	2.83
Serine	4.93	5.06	4.96	4.88	4.97	5.09	4.83	4.79	4.93	4.94
Glutamic acid	34.44	31.28	34.44	34.46	30.25	30.50	33.35	33.83	33.04	33.32
Proline	10.20	9.55	9.53	9.90	4.05	9.10	5.16	9.23	8.86	9.08
Glycine	3.48	4.09	3.51	3.57	4.27	4.17	3.58	3.53	3.49	3.48
Alanine	3.23	4.04	3.25	3.28	3.50	3.48	3.12	3.15	4.13	4.09
Half cystine	1.72	1.66	1.62	1.56	1.64	1.56	1.55	1.49	1.71	1.61
Valine	3.12	3.21	2.82	2.86	3.12	3.10	2.90	2.70	2.89	2.99
Methionine	1.51	1.50	1.47	1.52	1.50	1.50	1.50	1.41	1.56	1.56
Isoleucine	2.53	2.42	2.31	2.36	2.40	2.39	2.33	2.32	2.40	2.45
Leucine	6.31	6.40	6.10	6.19	6.25	6.30	6.16	6.12	6.29	6.29
Tyrosine	3.01	3.12	3.08	3.00	3.25	3.13	3.18	3.16	3.12	3.05
Phenylalanine	4.38	4.54	4.63	4.66	4.52	4.57	4.73	4.85	4.70	4.69
Histidine	3.19	3.20	3.35	3.51	3.50	3.47	3.48	3.64	3.44	3.41
Arginine	3.50	4.39	4.31	4.12	5.11	5.07	4.50	4.62	4.30	4.17
Lysine	2.51	2.92	3.00	2.76	3.54	3.41	3.20	2.83	3.35	3.05
Amino acid (g/100 g sample)										
Lysine total	—	—	0.31	0.26	0.42	0.39	0.31	0.25	0.35	0.31
Lysine available	—	—	0.29	0.24	0.39	0.37	0.29	0.23	0.33	0.29

^a Amino acid g/100 g protein (corrected to 100% recovery basis).^b WF indicates white flour; WWF, whole wheat flour; PCW and PCWB, pizza crusts prepared from white flour, unbaked and baked; PCWW and PCWWB, from whole wheat flour, unbaked and baked; PCC and PCCB, from commercial frozen pizza, unbaked and baked; and PCCM and PCCMB from commercial pizza mix, unbaked and baked, respectively.

ing in proteins is the water content of the system. Anhydrous protein is fairly stable to heat and storage in the presence of carbohydrate. At water activities of 0.4–0.7, the browning reaction proceeds rapidly. The browning reaction then slows as the protein is diluted. Liquid milk, therefore, is more stable to heating effects than powdered milk with residual moisture (Schroeder *et al.* 1953). Erberstobler (1970) studied the effects of moisture on available lysine in milk powder held for 10 weeks at 30°C. The results are presented in Table 19.8. These data illustrate the fact that moisture level is critical to the browning reaction.

TABLE 19.8. INFLUENCE OF MOISTURE CONTENT ON AVAILABLE LYSINE IN MILK POWDERS

Moisture in milk powder	Lysine (g/16 g N)	
		Available lysine
4		7.0
6		6.5
8		4.8
10		5.5

Kato *et al.* (1978) conducted an extensive study of the effects of the Maillard reaction on several attributes of egg whites. Egg whites were stored at a relative humidity of 65% at 50°C with and without glucose being present. It was observed that during the initial stages of the browning reaction the heat stability of the egg white protein actually increased. After 20 hr, the proteins became much more heat labile. Solubility went through a sigmoidal transition with an initial dip at 5 hr and a maximum solubility, then a decline to less than 25% solubility after 40 hr storage.

From the huge body of literature describing the browning reaction it is clear that heating and/or storage of protein in the presence of reducing sugars and limited water is an environment that will facilitate rapid degradation of the protein, particularly the ϵ -amino group.

PHOTOOXIDATION REACTIONS OF PROTEINS

Photooxidation of proteins is frequently ignored because it is relatively minor compared to the more dramatic effects discussed earlier in this chapter. Feeney (1980) discussed the possible photochemical

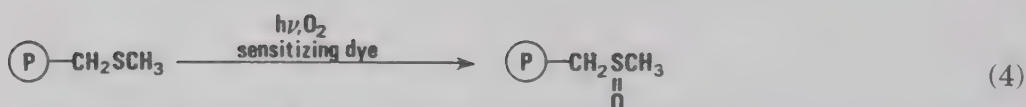
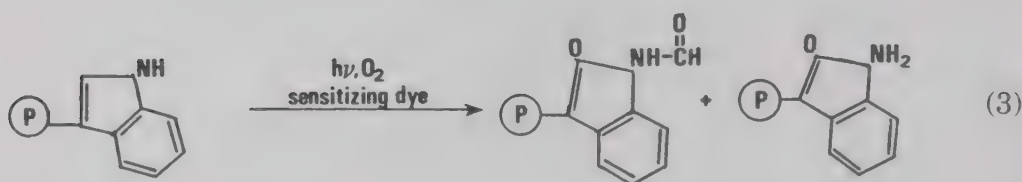
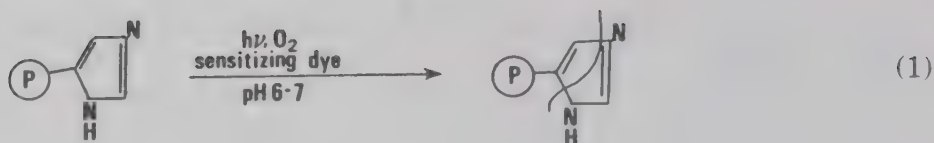


FIG. 19.5. Photooxidation pathways for histidine (1), tyrosine (2), tryptophan (3), and methionine (4). Wavy lines through structures indicate ring scissions. Cysteine is oxidized to cystine, in some cases, without sensitizing dye; cystine may also be oxidized.

From Feeney (1980).

reactions that could take place in proteins. These reactions are shown in Fig. 19.5. Amino acid side chains that are readily modified by photooxidation are sulfhydryl, imidazole, phenoxyindole, and thiol ether. Indicative of the reactions in Fig. 19.5 are the results in Table 19.9. The samples in Table 19.9 were taken from a sealed bottle of isolated soy protein which had been left on the laboratory shelf for 1 yr. The data indicate that there are losses in the oxidizable amino acids, but that aspartic acid and valine are stable to photooxidation. Possible photosensitizers might have been phenolics in the isolate, possibly acting in conjunction with oxidizing lipids. As with other degradative protein reactions, it is difficult to isolate the absolute cause of the decomposition.

Schaich (1980A) in a detailed review discussed the mechanisms of

free radical initiation and subsequent damage to protein which can result. Some of the basic mechanisms are summarized in Fig. 19.6. Although a number of alternative decomposition reactions exist, free radicals do play an important role in photolytic reactions both in direct dissociations and in photosensitized oxidations. Several factors influence the pathway and extent of free radical damage: (1) the nature of the sensitizer (photoreduced or producer of singlet oxygen); (2) the nature of the substrate and redox potential of the substrate; (3) concentration of sensitizer and oxygen in the system; and (4) the nature of the medium (potential for diffusion).

The aliphatic amino acids, although they absorb light only to a small extent, can be damaged significantly by the radiation in the absence of a photosensitizer. The damage results from short wavelengths. For

TABLE 19.9. EFFECT OF SAMPLING AREA WITHIN A BOTTLE^a ON AMINO ACID ANALYSIS OF ISOLATED SOY PROTEIN

Sampling area	Amino acid recovered/16 g N						
	Asp	Val	Tyr	Met	Cys	His	Trp
Top	10.03	4.31	3.30	1.15	1.02	2.70	1.30
Side							
(Exposed to light)	10.05	4.30	3.33	1.18	0.94	2.61	1.25
Middle	10.05	4.29	3.45	1.23	1.04	2.67	1.35

^a Sample bottle was exposed to fluorescent light for 1 yr sitting on laboratory shelf.

example, glycine is not damaged at wavelengths above 2265 Å. The precise changes and pathways of destruction are influenced by irradiation wavelength, irradiation dose, reaction conditions, and the individual amino acid being irradiated. The complete decomposition pathway has not been elucidated for any amino acid at this point (Weizmann *et al.* 1936; Vladimirov *et al.* 1970). Although damage to aliphatic amino acids has not been linked with inactivation of enzymes, evidence shows that there is damage by ultraviolet light. It has been proposed that glycine radicals are involved in the yellowing of wool (Collins and Grant 1969; Leaver and Ramsey 1969).

The sulfur amino acids, as indicated by the data in Table 19.9, exhibit more measurable photodecomposition than the aliphatic amino acids. Schaich (1980A) has summarized the photodecomposition reactions of cystine, as shown in Fig. 19.7. From the figure it can be seen that the decomposition of this simple amino acid is extremely complicated. The scheme in Fig. 19.7 summarizes the observations about the photodegradation of sulfur amino acids. The carbon-sulfur bond is the most labile (Forbes *et al.* 1962). This is supported by the fact that

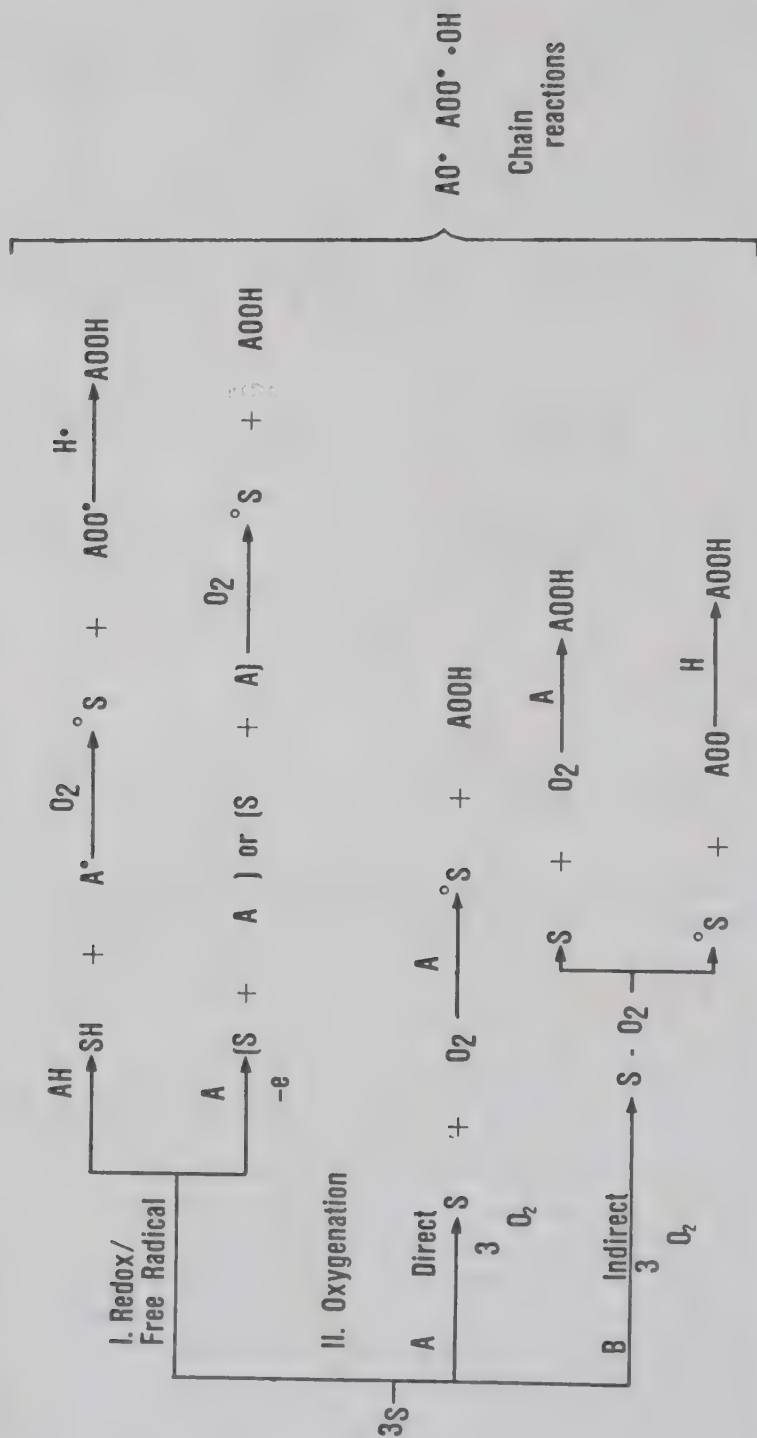


FIG. 19.6. Mechanisms of photosensitized oxidation. (I) Free radical or redox and (II) activated oxygenation, both direct and indirect. S is the mediating triplet sensitizer, and A is any acceptor molecule.
From Schaich (1980A, B).

alanine is the major reaction product after irradiation of either solid cystine or cystine in solution at 2537 Å (Forbes and Savage 1962). At wavelengths greater than 3000 Å, the carbon-sulfur bond is relatively stable and the primary products would be from oxidation.

The aromatic amino acids can act as photosensitizers in the protein, particularly sensitizing the sulfur amino acids. Pailthorpe and Nichols (1972) reported that aromatic amino acids in keratin photoejected electrons which attached themselves to adjacent sulfur amino acids. Table 19.10 lists some of the decomposition products that have been reported for the aromatic amino acids.

TABLE 19.10. PHOTODECOMPOSITION PRODUCTS OF AROMATIC AMINO ACIDS

Histidine	Phenylalanine	Tryptophan	Tyrosine
Aspartic	Aspartic	Aspartic	Aspartic
Glutamic-	Serine	Serine	Serine
hydroxy-	Tyrosine	Glycine	Glycine
glutamic	Alanine	Alanine-	Asparagine
Citrulline	Mono- or dihy-	(3-oxindolyl)-	Tyramine
	droxyphenyl-	alanine	Dopa
	alanine	Indole-3-acetic	p-Hydroxy-
	Ammonia	kynurenine	phenyl lactic
		N-Formyl-	melanins
		kynurenine	Ammonia
		Tryptamine	
		3-Hydroxy-	
		anthranilic	

Two of the potent photosensitizers in foods are riboflavin and chlorophyll. Generally, two classes of reactions are initiated when a photosensitizer, oxygen, and a protein are present and light of the proper wavelength activates the system. In the first type, the photosensitizer is excited to the triplet state by light of the proper wavelength. The excited sensitizer then univalently oxidizes an amino acid side chain of the protein initiating a free radical destruction of the amino acid. In the second type of reaction the excited photosensitizer excites a ground-state oxygen which forms a highly reactive singlet oxygen. The singlet oxygen can then attack lipids or reactive side chains of amino acids (Foote 1976).

INTERACTION OF PROTEIN WITH OXIDIZING LIPIDS

The chemistry of the oxidation of lipids is covered in several other chapters of this volume. Schaich (1980B) has also reviewed the chem-

istry of lipid oxidation and the transfer of free radicals to proteins from oxidizing lipids. In this section an attempt is made to deal with some of the products of lipid oxidation as reactants with protein. Figure 19.8 summarizes some of the reactions which can occur between protein side chains and amino acids. From Fig. 19.8 it is clear that the lipid hydroperoxides cause a number of interesting reactions with various reactive amino acid residues in proteins. These various reactions all help account for the polymerization of proteins reported by Roubel and Tappel (1966A), where severalfold increases in the molecular weight of protein were observed. It was suggested that the lipid peroxidation free radicals served as initiators of the polymerization. Roubal and Tappel (1966B) also reported substantial losses in amino acids when proteins were exposed to peroxidizing lipids. They observed that methionine, histidine, cystine, and lysine were the most vulnerable to damage. Horigome *et al.* (1974) reported the oxidation of casein and ovalbumin with oxidizing ethyl linoleate, although the losses in amino acids were generally less than in the earlier study. Horigome *et al.* (1974) also observed losses in digestibility and biological value of the proteins after oxidation. Because methionine is the first limiting amino acid in most noncereal plant foods, sulfur amino acid availability is an issue of some concern. Tannenbaum *et al.* (1969) reported that methionine residues in protein were oxidized to methionine sulfoxide in the presence of oxidizing lipid. Cuq *et al.* (1973) found the same oxidation in proteins treated with hydrogen peroxide. Finley and Lundin (1980) reported oxidation of cystine residues to a variety of sulfoxides and sulfones, as are shown in Fig. 19.8. Tufte and Warthesen (1979) observed considerable conversion of methionine to methionine sulfoxide when methionine is exposed to oxidizing lipid in a model food system. Tannenbaum *et al.* (1969) studied the loss of methionine in casein which was exposed to autoxidizing methyl linoleate in a model system. They also observed that considerable browning takes place in the model system as the reaction continues. Table 19.11 contains the bioavailabilities of the various oxidized sulfur amino acids. There appears to be some confusion about the availability of methionine sulfoxide with values ranging from 50 to 100%. Crawford *et al.* (1984) reported the values for the cysteine/cystine oxidation products and concluded that the availability of cystine oxidation products was based on the oxidation state of the sulfur. When the amino acid sulfur is oxidized to the valence of four the amino acid remains available, but when the valence is raised to six it becomes unavailable. Snow *et al.* (1975) reported the reduction of methionine sulfoxide to methionine by cysteine. Based on this kinetic study we would speculate that much of the variation in results on the avail-

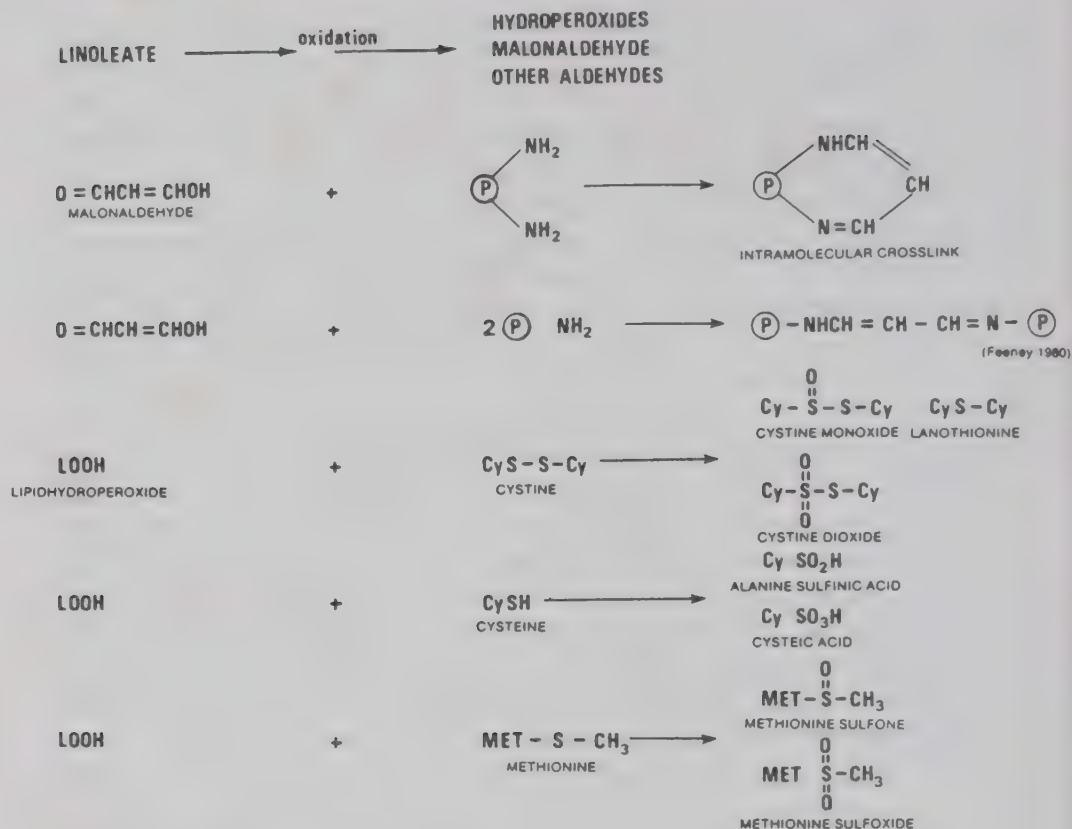


FIG 19.8. (Continued on page 467).

ability of methionine sulfoxide may be based on the availability of sources of cysteine in the diet which could modify the methionine sulfoxide before ingestion. In addition, the overall oxidative condition of the diet could affect the methionine sulfoxide. In our laboratory we have also observed that methionine sulfoxide disproportionates to yield methionine and methionine sulfone on standing. This observation has never been confirmed in diets, but should be considered.

Malonaldehyde, an intermediate in the decomposition of lipids, has been shown to react with sulfur amino acids, with enamine and imine linkages being proposed in the products (Aray *et al.* 1972). Later Svanlenka *et al.* (1975) reported that when malonaldehyde reacts with collagen, lysine and tyrosine are the principal amino acids damaged. Szebiotko *et al.* (1979) studied the interactions of proteins with oxidizing lipids and reported a number of interesting observations about the interactions. From their work it appeared that maximum interaction or degradation of the protein takes place when the lipid oxi-

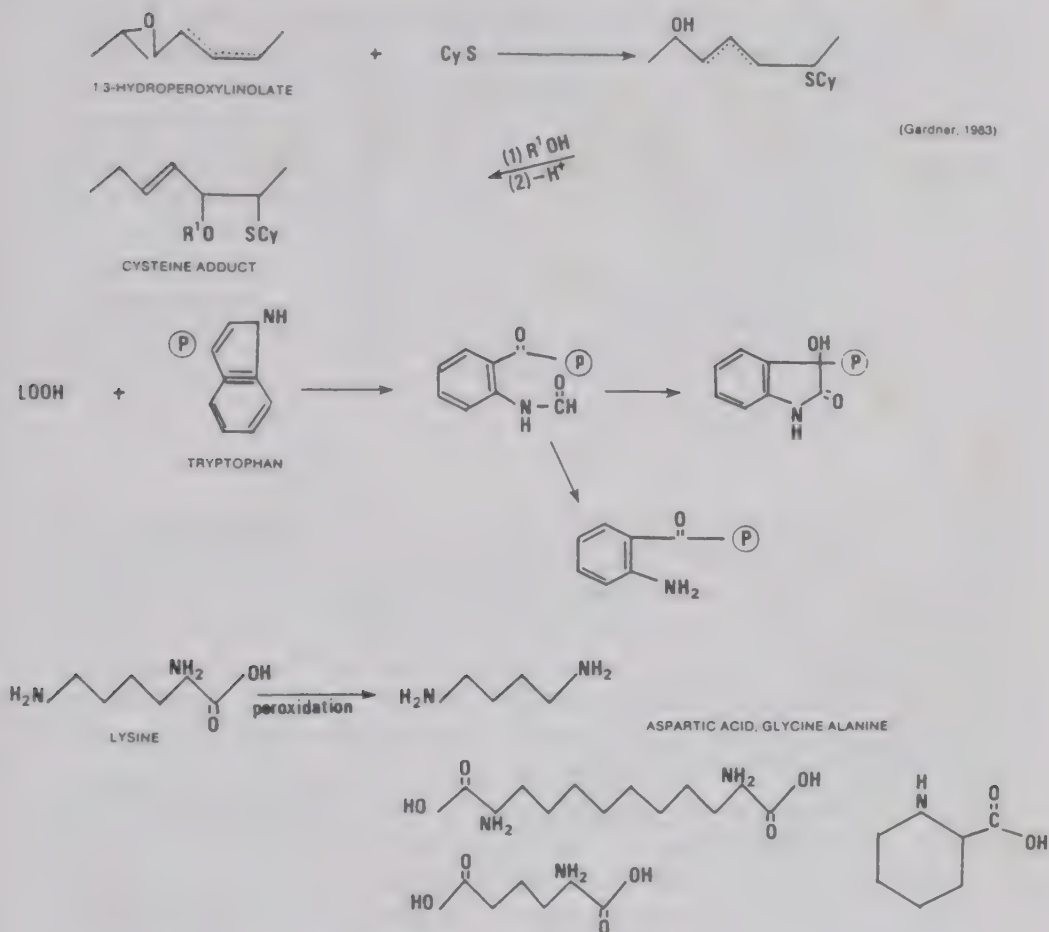


FIG. 19.8. Interactions between oxidizing lipids and protein or amino acids.
From Karel et al. (1975).

dation is at the stage of maximum peroxide formation. Losses in available lysine appeared to take place in the initial induction period and during the induction of peroxides. Losses in available lysine then appeared to show less change with time at later stages of the oxidation. Tryptophan losses were also seen predominantly during the maximum peroxide period. All of these results serve to underline the importance of peroxidation on the radicals generated during that stage in damage to protein.

Oxidizing lipids or peroxides in the environment of the protein clearly cause significant change in the protein. The oxidations and cross-links generated tend to adversely affect solubility, enzyme activity, and nutritive quality of the protein.

Chlorine is another environmental oxidizer which can damage pro-

TABLE 19.11. AVAILABILITY OF THE SULFUR AMINO ACIDS

Amino acid	Availability in chick (%)
Methionine	100
Methionine sulfoxide	50-100
Methionine sulfone	0
Cysteine	100
Cystine	100
Cystine monoxide	100
Cystine-S-S'-dioxide	93
Cystine-S-S-dioxide	51
Alanine sulfinic acid	0
Cysteic acid	0

From Crawford *et al.* (1984).

tein quality. Urabe *et al.* (1975) studied the chlorinolysis of methionine in a model system. The reaction resulted in the formation of polychloroamino acid derivatives. The significance and extent to which these derivatives are formed in chlorinated foods such as flour is unresolved at this time. The initial site of attack of the chlorine is the sulfur of methionine. The first intermediate formed in the reaction appears to be a chlorosulfonium salt. The second step in the reaction is the formation of a carbonium ion intermediate and a cleavage of the carbon sulfur bond. The splitting yields a trichloroamino acid product. The relationship of the chlorination of methionine to the changes in flour functionality due to changes in the structure of the peptide is unknown. In reality, the nutritional impact is not likely to be significant, since foods produced from chlorinated flour are not generally consumed as sole sources of protein; therefore, the loss of small amounts of methionine would not be significant.

INFLUENCE OF ALKALINE CONDITIONS ON PROTEIN

Proteins, particularly purified proteins, are frequently exposed to a high pH environment. Even a brief exposure of protein to an extreme in pH can result in significant changes in the protein. Alkaline treatment has the obvious advantage of improving solubility of protein, destroying toxins, or improving flavor or texture of the protein (Circle and Smith 1972; Hermannsson *et al.* 1971; Shetty and Kinsella 1980; Tannenbaum *et al.* 1970; Betschart 1974). The undesirable aspect of alkali, particularly at high temperatures, is that racemization and new undesirable cross-links such as isopeptides or lysinoalanine can

be formed. The formation and influence of isopeptides was discussed earlier under the section on effects of heat on protein. Otterburn (1983) has reviewed the formation of isopeptides under a variety of environmental conditions. Emphasis here will be on the formation of lysinoalanine and similar cross-links which are more readily formed under alkaline conditions. Finley (1983) reviewed the chemistry of lysinoalanine formation in detail, so for the purposes of this chapter the chemistry will only be highlighted.

Lysinoalanine and related cross-links share a common starting point which is the β -elimination reaction outlined in Fig. 19.9. The β -elimination reaction results in the formation of dehydroalanine. Dehy-

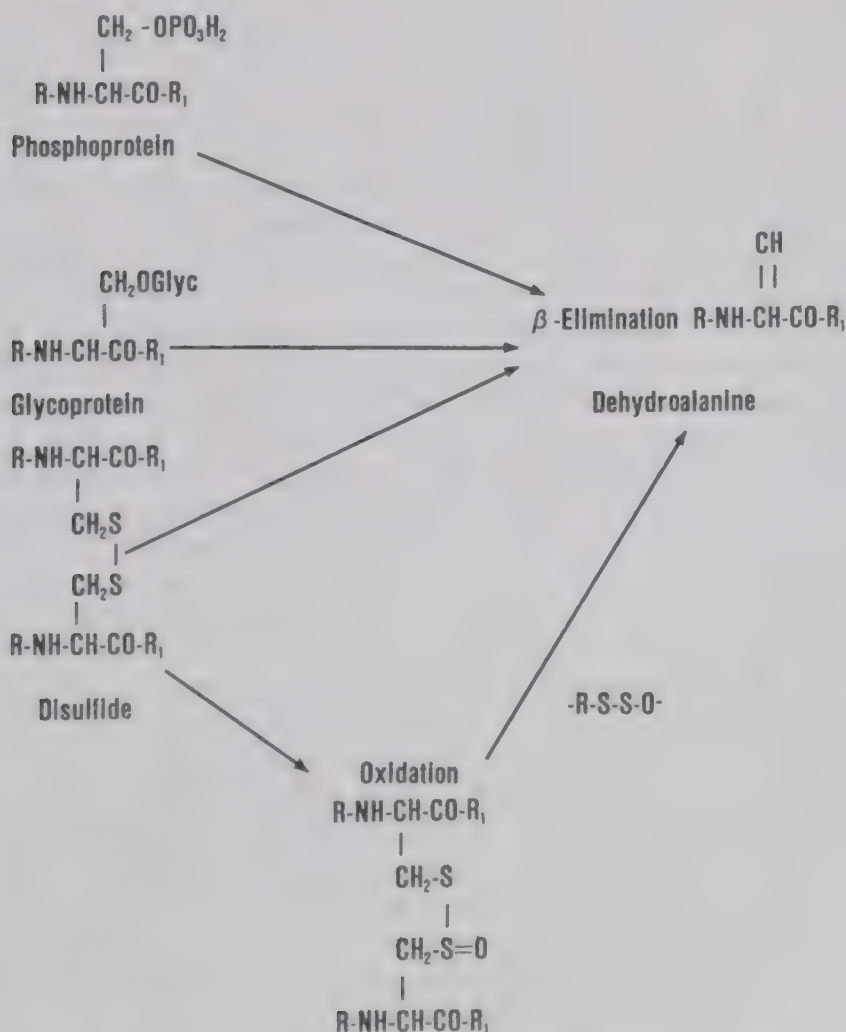


FIG. 19.9. Postulated pathways for the formation of dehydroalanine in proteins.

dehydroalanine can be formed from a variety of amino acids found in protein. Casein has been shown by several workers to form high levels of lysinoalanine (Finley 1983). Dehydroalanine is an intermediate in the formation of lysinoalanine. A significant portion of the serine in casein is present as phosphoserine. Phosphoserine has been shown to undergo β -elimination at a very rapid rate, thus accounting for the ease of dehydroalanine formation in casein (Whitaker and Feeney 1977). It also has been shown that calcium enhances the β -elimination reaction (Sen *et al.* 1977), since casein is likely to contain high levels of calcium which increases its sensitivity. As with other reactions discussed in this chapter, it is frequently difficult to separate the reactions from one another. The effects of alkali on the disulfides of protein were studied by Nashef *et al.* (1977), and mechanisms for β -elimination of sulfur amino acids are discussed in detail. Oxidations are known to proceed more rapidly in alkaline environments. It has been reported that oxidation of the cystine residues in a protein greatly enhances the β -elimination reaction (Finley *et al.* 1982). Fujimaki *et al.* (1980) reported that when proteins are oxidized with performic acid to oxidize the cystine to cysteic acid, much more rigorous alkaline treatment is required to observe lysinoalanine formation. This is very consistent with the results of Finley *et al.* (1982) where it was observed that the more highly oxidized cystine oxidation products (alanine sulfinic acid and cysteic acid) were slower to undergo β -elimination than the partially oxidized intermediates (cystine, cystine monoxide, and cystine dioxide). Isolated soy protein also forms lysinoalanine readily, and it would appear that the reaction is influenced by the oxidation state of the system, the air incorporation during processing, the ionic strength, the temperature, and the pH.

The dehydroalanine formed in a protein is very reactive and can react with a number of side chains of the protein. Figure 19.10 summarizes some of these reactions which have been reported. A thorough discussion of the Michael addition reactions can be found in work by Friedman (1977), Asquith *et al.* (1974), and Fearheller *et al.* (1977).

Figure 19.10 illustrates some of the typical reaction products that are formed as a result of the addition reaction of dehydroalanine with side chains found in proteins. In addition to the reactions illustrated here, it would seem conceivable that dehydroalanine could cross-link with reactive amines in nucleic acids to yield nucleic acid protein cross-links. Lanthionine and lysinoalanine appear to be the most frequently observed cross-links in practice; however, in severely treated materials ornithinoalanine does appear.

The overall effects of processing conditions on lysinoalanine formation have been investigated in a wide variety of proteins and foods.

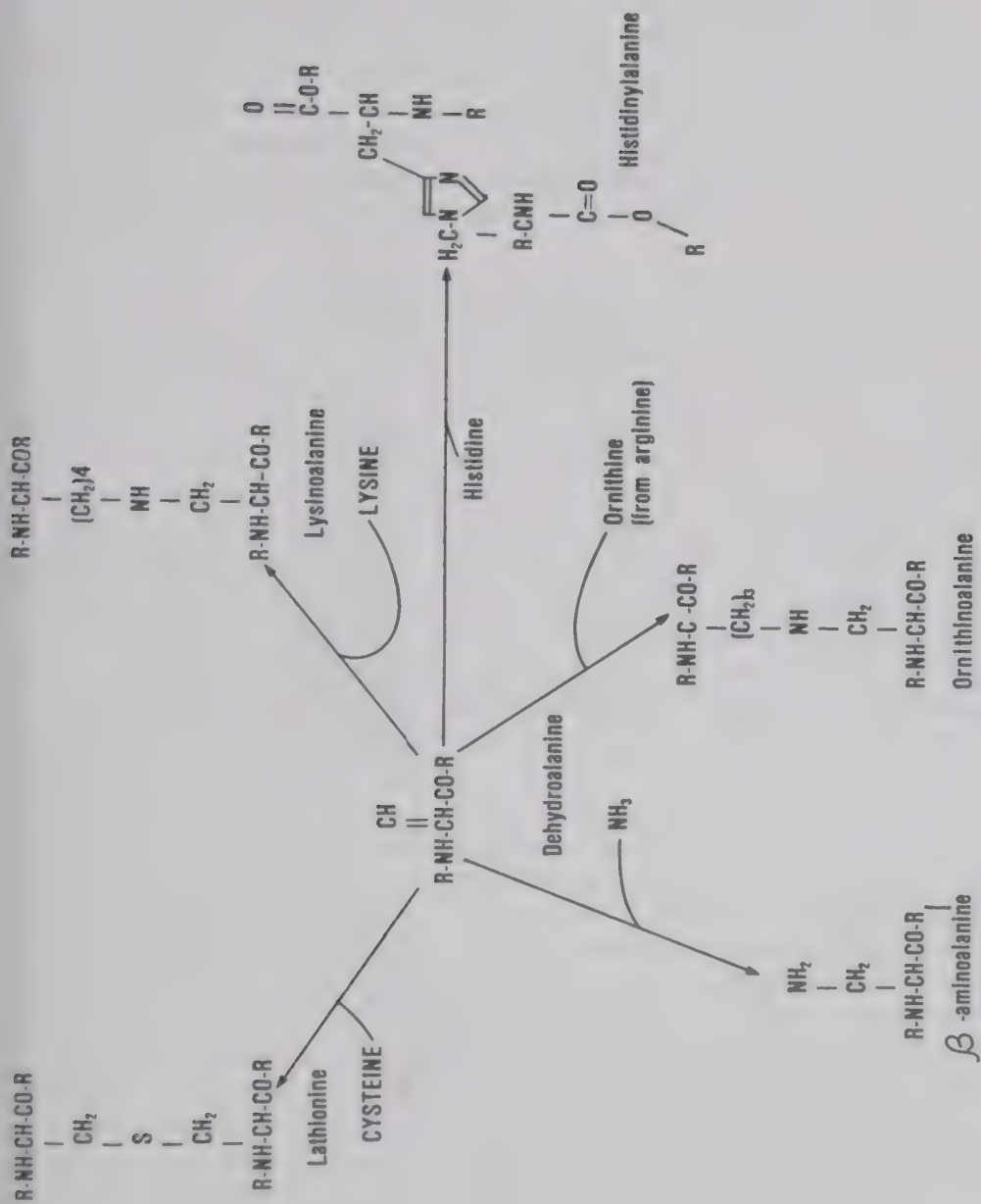


FIG. 19.10. Reaction products between dehydroalanine and various side chains in proteins.

Hayashi and Kameda (1980) investigated a broad range of conditions which influence the formation of lysinoalanine in pure proteins. Generally, the cross-linking increases with increases in time, temperature, and pH, reaching a maximum value for the individual protein. Fujimaki *et al.* (1980) found similar trends in wheat gluten and fish protein. Aymard *et al.* (1978) reported the presence of lanthionine and lysinoalanine in several food proteins. It was concluded that intense heat at neutral pH could also result in limited formation of lysinoalanine. Table 19.12 summarizes typical lysinoalanine levels that have been reported in a number of pure proteins and some food proteins.

TABLE 19.12. LYSINOALANINE IN VARIOUS FOOD PROTEINS

Protein source	Lysinoalanine (ppm)	Reference
Lysozyme	490	Bohak (1964)
Papain	241	Bohak (1964)
Phosvitin	830	Bohak (1964)
Lysozyme	670	Finley <i>et al.</i> (1982)
Bovine serum albumin	240	Finley <i>et al.</i> (1982)
Sodium caseinate	100–1400	Erberstobler and Holstein (1980)
Sodium casinates	80–970	DeKoning and Rooijen (1982)
Potassium caseinate	<25	DeKoning and Rooijen (1982)
Calcium caseinate	0–2820	DeKoning and Rooijen (1982)
Hominey	0	Sanderson <i>et al.</i> (1978)
Masa	200	Sanderson <i>et al.</i> (1978)
Soy isolate	20–2000	Aymard <i>et al.</i> (1978)
Soy isolate	<5–75	Finley (1984)

From the table it can be seen that in alkaline-treated proteins there can be significant levels of lysinoalanine produced. Walsh *et al.* (1979) studied the cross-linking of ovomucoids in alkali. The careful study followed the losses of lysine and the appearance of lanthionine in the model system. Generally, however, it would seem that in normal food processing the levels are modest. Lysinoalanine does serve as an excellent marker for severely treated proteins. When proteins are exposed to an alkaline environment, lysinoalanine serves as an excellent analytical indicator of too severe a treatment. The soy isolate data in Table 19.12 clearly indicate that commercial soy isolate can be produced with less than 50 ppm lysinoalanine.

The biological effects of lysinoalanine and alkaline-treated protein have been reviewed by Finley (1983). Toxic effects of alkaline-treated casein and fish protein in chicks were reported by Carpenter and Duckworth (1950). Later work has pointed out the difficulty in separating the effects of lysinoalanine and racemization on toxicity and

protein quality. Several workers have reported kidney lesions resulting from rats being fed proteins containing lysinoalanine. Newberne and Young (1966) observed microscopic kidney lesions when rats were fed alkaline-treated soy. Woodard's group (Woodard and Alvarez 1967; Woodard 1971A, B; Reyniers *et al.* 1974; Woodard *et al.* 1975; Woodard and Short 1977) confirmed that the renal lesions were in pars recta of the proximal tubule of the kidney. The lesion was attributed to lysinoalanine in the diet and was completely described by Woodard *et al.* (1975). De Groot *et al.* (1976) reported feeding lysinoalanine to several species and concluded that only the rat was susceptible to the lesion. Gould and MacGregor (1977) proposed several nutritional factors which could influence the susceptibility of an animal to lysinoalanine toxicity. The amino acid profile of the protein appeared to be very important, with alkaline-treated soy isolate inducing more changes than lactalbumin which contained more lysinoalanine. Methionine level, salt content of the diet, and carbohydrates all seemed to be influential on the number of lesions observed. Hayashi (1982) has suggested that the chelating characteristics of lysinoalanine could be involved in its toxicity. Lysinoalanine could potentially remove metal ions from metallo enzymes or compete with the metallo enzymes for metal ions, thereby inhibiting the enzyme. Inhibitions of carboxypeptidase β and alcohol dehydrogenase were demonstrated as examples.

From the current literature it appears that free lysinoalanine is significantly more toxic than peptide-bound lysinoalanine. Mild alkaline treatments as used in the food industry do not appear to yield toxic levels of lysinoalanine.

In addition to β -elimination and subsequent cross-linking which can occur in alkaline-treated proteins, racemization also can and does occur when proteins are exposed to an alkaline environment. Here again is a situation where the influence of the two effects on protein digestibility and availability cannot be completely separated, since it is difficult to cause one effect in a protein to the exclusion of the other.

Racemization was first observed in proteins about 75 years ago (Kossel and Weiss 1909) in severely alkaline-treated proteins. Neuberger (1948) proposed a base-catalyzed racemization reaction which is illustrated in Fig. 19.11. The reaction occurs via removal of the α -methine hydrogen, forming a carbanion intermediate. The carbanion then reacts rapidly with a proton with an equal likelihood of readdition of the proton forming either the normal L form or the D form of the amino acid residue. Protein sequence may have some mediating influence on this at moderately alkaline pH, but as pH increases, the randomness of the protein structure would also be expected to increase. The rate of racemization is proportional to the hydroxide ion

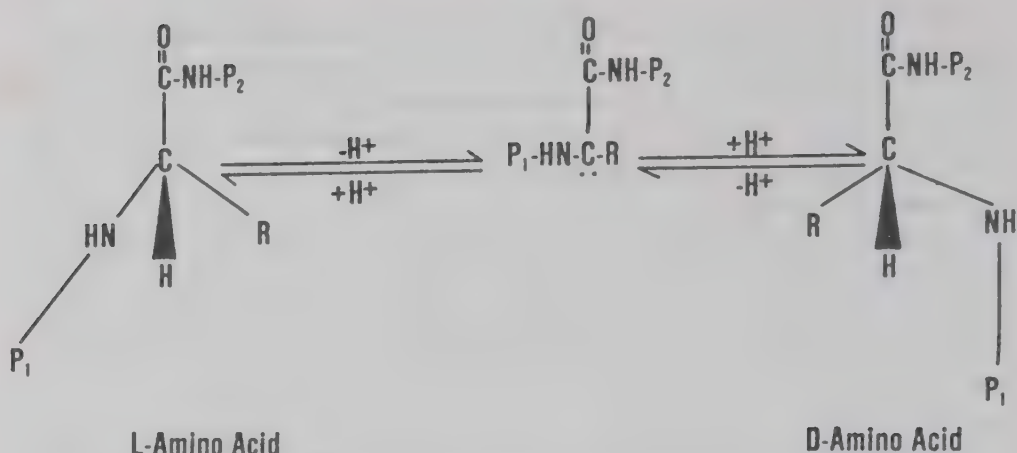


FIG. 19.11. Pathway for racemization of amino acids in proteins. P_1 , Portion of protein chain containing the amino terminus group; P_2 , portion of protein chain containing the carboxyl terminus.

concentration above pH 8.0 (Bada 1972). Below pH 8.0 racemization rate is dependent on electron withdrawing ability or the amino acid side chain. Under strongly acid conditions, below pH 1.0, acid-catalyzed racemization occurs (Manning 1970). In addition to high pH, increases in length of alkaline treatment and temperature increase racemization (Friedman *et al.* 1981). Heat at neutral or slightly acid pH can induce significant racemization. Hayase *et al.* (1973, 1975) roasted several proteins and poly-L-amino acids. In this work glutamic and aspartic acids were the most easily racemized. Bjarnason and Carpenter (1970) observed that in heated bovine plasma and cod fillets (145°C) there was considerable racemization.

Early work (Dakin 1912; Levene and Bass 1929) indicated that free amino acids and dipeptides were more resistant to racemization than protein. Whitaker (1980) proposed that amino acid residues in proteins undergo more racemization than free amino acids because the increased electron density around the free amino and carboxyl groups interferes with the abstraction of the proton from the α -methine. The position of the amino acid residue in the peptide or protein can also have a major influence on the tendency to racemize (Smith and deSol 1980).

Several laboratories have documented the extent to which racemization occurs in alkaline-treated proteins (Hayashi and Kameda 1980; Tannenbaum *et al.* 1970; Provansal *et al.* 1975; Friedman *et al.* 1981). Tovar and Schwass (1983) have reviewed the influence of the type of alkali on racemization in proteins, particularly zein. Table 19.13 con-

tains data on the extent of racemization of a variety of proteins which have been exposed to alkaline conditions and includes data on lysinoalanine formation in the same proteins. It also illustrates that some commercial foods do have modest levels of racemization of amino acids in proteins, but the levels are generally low. One exception, however, is the one liquid diet. The commercially available weight loss formulation was presumably treated with too much alkali in error and was released for sale. The levels of D-serine in this sample could be a hazard if it were consumed as sole protein source for an extended period. Hayase *et al.* (1979) observed that roasting protein in the presence of methyl linoleate resulted in substantial racemization of the amino acids in the protein at neutral pH. It was observed that normal components of a food system such as glucose and lipids greatly enhanced the extent of racemization in the roasted proteins.

The biological effects of racemization in proteins has been studied extensively. Several workers have reported marked decreases in the *in vitro* digestibility of alkaline-treated proteins. Dakin (1908) demonstrated that alkaline-treated casein was much more resistant to enzymatic hydrolysis than untreated casein. The cross-linking that occurs during alkaline treatment makes study of racemization where there is no cross-linking extremely difficult. In an attempt to resolve this issue, Tovar (1981) studied alkaline-treated zein, which because it lacks lysine should not form lysinoalanine. In the study, zein which had been treated with sodium hydroxide and zein treated with lime

TABLE 19.13. RACEMIZED AMINO ACIDS AND LYSINOALANINE IN FOOD PROTEINS

Protein	Ileu	Leu	Pro	Thr	Val	Ala	Glu	Lys	Phe	Asp	Ser	Lal (ppm)
Liquid diet	0	0	3.8 ^a	0	0	2.4	4.4	0	2.0	24.2	0	0
Liquid diet	0	0	2.3	0	0	2.3	4.7	0	0	25.4	3.7	0
Liquid diet	0	0	3.4	2.0	0	2.6	4.8	0	4.2	19.9	4.4	0
Liquid diet	18.4	1.2	3.3	4.1	0	2.2	4.2	1.8	4.2	14.2	0	0
Liquid diet	18.1	67.3	0	7.0	7.7	26.0	27.4	22.7	26.0	37.1	49.9	55
Wheat crackers	0	3.9	3.7	0	0	8.5	5.7	0	3.9	9.5	0	0
Wheat cereal	0	0	3.0	0	0	0	3.7	0	3.1	11.0	0	0
Hominey	0	3.5	3.6	0	0	0	6.9	0	2.9	15.4	0	0
Tortilla	0	0	2.9	0	0	0	7.6	0	2.5	11.6	0	0
Soy isolate	3.2	3.5	4.3	0.8	1.4	3.3	4.5	2.9	2.9	8.2	0.8	75
Soy isolate	0	0	3.3	0	1.1	2.4	3.8	0	2.6	7.1	0	25
Soy isolate	0	0	3.3	0	1.0	2.0	2.3	0	2.3	7.5	0	20
Infant formula	0	0	0	0	0	0	2.9	0	0	7.3	0	0
Infant formula	0	0	0	0	0	0	3.3	0	0	8.2	0	0
Infant formula	0	0	3.2	0	0	1.9	3.6	0	2.1	9.0	1.6	Tr

^a Percentage D-amino acid.

were compared. Rats fed the sodium hydroxide-treated zein exhibited diarrhea, alopecia, and poor weight gain compared to control animals and animals receiving the lime-treated zein. Both alkaline-treated zeins exhibited equivalent levels of racemization. In a related study, Schwass *et al.* (1983) observed reduced uptake by perfused jejunum of amino acids from pronase digests of the alkaline-treated proteins. Bunjapamai *et al.* (1982) studied the *in vitro* digestibility of alkaline-treated casein, some of which had lysinoalanine formation and some of which did not. The digestibility of the two alkaline-treated caseins that exhibited racemization was the same whether or not lysinoalanine were present in the protein. Both treated proteins had lower digestibility than the untreated control.

Tovar and Schwass (1983) reviewed the availabilities of D-amino acids to humans, rats, and chicks. It was concluded that the D-forms of most of the essential amino acids are not utilized by humans. Therefore, it would seem that the environments of proteins should be carefully controlled to reduce the chance of formation of D-isomers. The data also suggest that lysinoalanine may be a convenient indicator for alkaline damage to protein.

CONCLUSIONS

Proteins can be severely damaged when exposed to harsh chemical or physical environments. The degree of damage varies with the intensity of the particular treatment. Since most of the treatments discussed here are interrelated, it is virtually impossible to isolate any single effect in a complex biopolymer such as a protein. It should also be recognized that most industrial processing conditions are milder than those considered here and do not cause the extent of damage observed in model systems studies. However, compounds resulting from treatments such as those described here may serve as indicators of excessive treatment.

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Environmental Effects on Chemical Changes in Foods

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INTRODUCTION

Foods are susceptible to quality losses due to chemical reactions, many of which are described in this volume. The rates of these reactions depend both on compositional and environmental factors. Control of undesirable changes during processing requires a quantitative analysis based on knowledge of kinetics of food deterioration.

Quantitative analysis of quality losses in any product during preparation, processing, and storage requires certain initial assumptions as well as a set of real or assumed data (Karel 1983). These assumptions include the existence of a measurable index of deterioration, that is, a chemical, physical, or sensory measurement or set of measurements which may be used reproducibly to assess the changes occurring in processing. This index has to be sensitive enough to express the effect of the environmental factors on the quality composition. For instance, nonenzymatic browning starts only after a certain lag period (Saguy *et al.* 1979). During this period the process effect on the quality loss is not obvious. Hence, if browning is chosen as the index of deterioration during this initial period, the quality losses would seem negligible, yet the damage will appear only at later states. This simple example indicates the necessity to choose the proper index

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of deterioration before any prediction of quality losses is attempted.

The index of deterioration expresses a chemical entity or a physical measurement whose loss or increase correlated with quality. The quantitative analysis of quality changes requires that the index of deterioration be expressed as a function of the conditions during preparation, processing, and storage in a manner which allows prediction or simulation of these changes. It is therefore normally accepted that calculation and prediction of quality losses require a kinetic/mathematic model that expresses the effect of the environmental and composition factors on the deterioration index. The model is often a set of nonlinear, coupled, partial differential equations, the analytical solution of which is either nonexistent or very complicated (Churchill 1974).

Progress has been made primarily by simplifying these equations for specific conditions. The kinetic approach is based on relating reaction rates to environment factors (temperature, pressure, etc.) and composition factors (concentration, pH, etc.).

The general equation describing quality loss may be given as

$$-dC/dt = f(E_i, F_j) \quad (1)$$

where $-dC/dt$ is the rate of deterioration, E_i is the environmental factor ($i = 1, \dots, n$), and F_j is the composition factor ($j = 1, \dots, m$).

The effects of some of these factors can be expressed relatively easily in a functional relationship that is similar for many deterioration reactions and food systems. Others are more complicated and unique in their behavior and must be derived separately for each product and food system investigated.

Quality of foods and the rate of quality changes in processing and storage depend obviously on the chemical composition. In many cases it is possible to correlate quality losses with the loss of a particular component, such as a vitamin or a pigment. In these cases the rate of the quality loss is represented as proportional to the power of the concentrations of the reactants:

$$-dC_i/dt = k C_1^{n_1} C_2^{n_2} \dots \quad (i = 1, \dots, m) \quad (2)$$

If $n_i = 1$, the reaction is first order with respect to C_1 . The overall reaction order n is the sum of the exponents ($n = \sum n_i$). The rate constant k thus has the units of $C^{1-n} \text{ time}^{-1}$. The value of n can be a fraction or a whole number. The order is strictly an empirical concept. However, when the stoichiometric equation represents the mechanism, the reaction order and the molecularity have the same value.

ENVIRONMENTAL EFFECTS ON RATES OF CHEMICAL REACTION

General Approach

The usual procedure in incorporating the effects of environmental factors in the kinetic equations such as Eq. (2) is to develop relations between the rate constant k and the various environmental factors:

$$k = f(T, a, O_2, \dots) \quad (3)$$

where T is the temperature, a is the water activity, and O_2 is the oxygen concentration.

Because temperature is an extremely important factor in all chemical reactions, and because temperature control is a major feature of most food processes, its effect on chemical changes in foods has been

TABLE 20.1. ROLE OF ENVIRONMENTAL FACTORS IN MAJOR CHEMICAL REACTIONS IN PROCESSING FOODS

Reaction	Key environmental variables affecting the reaction
I. Nonenzymatic browning	Temperature, water activity
II. Lipid oxidation	Temperature, water activity, composition of gaseous phase
III. Cross-linking of polymers	Temperature, water activity
IV. Enzymatic changes in refrigerated fruits and vegetables	Temperature, composition of gaseous phase
V. Oxidation of meat pigments	Temperature, composition of gaseous phase

studied very extensively, and kinetic relations involving temperature are well developed. Some progress has also been made in relating kinetics of chemical changes to water activity and to gas composition of the atmosphere. Other environmental factors are less well developed. Important environmental factors affecting major chemical reactions in foods are listed in Table 20.1.

Temperature Effects

To relate the rate of quality loss to temperature, the most common and generally valid assumption is that temperature dependence of the given deterioration reactions will follow the Arrhenius equation:

$$k = k_0 \exp(-E_a/RT) \quad (4)$$

where k is the rate constant, k_0 the constant, independent of temperature (also known as preexponential frequency factor or collision factor), E_a the activation energy, R the ideal gas constant, and T the absolute temperature.

The activation energy is generally derived from the slope of the plot of $\ln k$ vs $1/T$ and depends on factors such as water activity, moisture content, solid concentration, pH, and others. Furthermore, when the reaction mechanism changes with temperature, the activation energy may vary substantially. Therefore, all equations, including the Arrhenius relation, have limited applicability (Tannenbaum 1975). Table 20.2 summarizes typical values of activation energies for different chemical reactions in food systems.

TABLE 20.2. TYPICAL ACTIVATION ENERGIES FOR REACTIONS IMPORTANT IN FOOD DETERIORATION

Reaction	Activation energy (kcal/mol)
Diffusion controlled reaction	0–8
Lipid oxidation	10–25
Nonenzymatic browning	25–50
Enzyme reactions	10–30
Off-color in dry vegetables	16–35
Off-flavor in dry vegetables	10–25
Vitamin loss	20–30

Recently, the Arrhenius equation was the topic of statistical analysis (Lund 1983). This analysis is most important since small errors in conducting the tests may be magnified if long extrapolations are utilized. Extrapolations are sometimes applied to predict shelf life of products stored at low temperatures based on kinetic data derived at elevated accelerated conditions.

Davies and Hudson (1981) suggested a simple way of reducing the high correlation between E_a and k_0 to acceptable levels by a simple transformation of Eq. (4). Thorough review of reaction kinetics as a function of temperature and of methods of simulation of the course of reaction under changing temperature conditions was presented recently by Labuza and Kamman (1983).

Water Activity and Water Content

The mobility of food components is greatly affected by the presence of water, since water is the most important solvent and plasticizer for the hydrophilic food components. The mobility of food components in

turn affects the physiochemical and physical properties of foods. The importance of water in food derives from this solvent and plasticizing power, and from the fact that water as a volatile and practically ubiquitous component of the atmosphere is capable of rapid equilibration between various phases. Since the driving force for this equilibration is the difference in chemical potential between different phases, and since at constant temperature the chemical potential is proportional to water activity, it is the water activity that determines the extent to which the system properties are affected by water.

Since the mobility of reactants and of products of chemical reactions is a key factor in rates of these reactions, it is obvious that water activity has an important impact on chemical changes in foods. The following features of this dependence on water activity are prominent (Duckworth 1981; Simatos *et al.* 1981; Karel and Flink 1983):

1. The first layer of water bound to food constituents is devoid of solvent properties (but some intramolecular mobility may be possible in this range).

2. Water above this layer is capable of solvent action, especially for small molecules.

3. When solute goes into solution, water activity of the system is depressed in accordance with the laws governing vapor pressure depression by solutes (Raoult's law).

4. Chemical reactivity is correlated with dissolution of reactants.

5. There is a characteristic water activity for dissolution of a given solute which is the same for different macromolecular systems in which the solute is dispersed.

6. Above a critical activity at which the given solute goes into solution there is a region of water activity in which there is partition between solute in a solid-like state and a dissolved state. There is, therefore, a heterogeneity of environments within this water activity level.

Effects of Water Activity on Enzymatic Activity

Critical Water Activity for Onset of the Reaction. Kertesz (1935) was one of the first to recognize that there was a "critical water concentration" for the activity of enzymes. He measured the hydrolysis of sucrose in model systems containing ground sucrose, apple pomace, and invertase mixed directly with various amounts of water and found that (1) no reaction occurred below 4.75% moisture from -18° to $+40^{\circ}\text{C}$, and (2) the rate increased with moisture content. Volgunov (1948)

followed the action of proteolytic enzymes on peptones dispersed on filter paper.

"Capillary water" hypothesis was advanced by German workers studying a variety of enzymes in the 1950s. Kiermeier and Coduro (1954) found that no reaction could be detected below a water activity (a_w) of 0.7 in a system consisting only of starch and amylase, but in a more porous matrix the onset of reaction was lowered to $a_w = 0.45$. They postulated that capillarity had a major effect on such reactions. Acker (1962, 1969) reviewed the studies of his own group and those of other investigators. Initially, they came to the conclusion that for any reaction to occur in dehydrated systems, "freely mobile capillary-condensed water" was required and that its function as a reactant (in hydrolysis) was secondary. Their preoccupation with "capillarity" led them to designate an inflection point of the isotherm as the cutoff point for enzyme-mediated reactions. Water below this point was considered too tightly bound to participate. The existence of capillaries was also used to explain steric effects. Subsequent studies led Acker's group to extensive revision of the earlier hypothesis (Acker and Beutler 1966; Purr 1966).

Another group of workers concerned with mechanisms of enzyme action at low levels of hydration was that of Drapron in France. Basing their conclusions on studies in model systems containing enzymes, substrates, and a variety of water-binding agents, they developed a theory of reactions at low hydration in which the onset of the reaction is ascribed to a water level at which solvent water begins to be available in a given system (Drapron 1972). Their experimental definitions of "solvent water," however, was based entirely on the *shape* of water sorption isotherms and are therefore quite imprecise.

Extent of Reaction. A number of workers, including those in the group of Acker (1969) and Drapron (Drapron 1972), observed that in low moisture systems, in contrast to dilute solutions, the reaction does not appear to progress toward a depletion of the substrate, but reaches a conversion extent "plateau." Acker (1962) explained this effect by the enzyme activity being limited to only those pores that can support condensation of bulk water at the specific water activity tested. He suggested that "as the relative humidity is increased, increasingly larger pores are filled so that an increasing amount of substrate can be dissolved and brought into reaction with the enzyme" (Acker 1962).

The French workers (Drapron 1972) considered the amylolysis in their system to be a topochemical degradation with very little mobility of the components, which appears to agree in large measure with what Acker (1969) hypothesized. Both groups concluded that the rate

of reaction at the site of each active enzyme is so much faster than the diffusion of the components involved that at each a_w the reaction appears to stop either because the local concentration of substrate is depleted or because the product concentration reaches that which is necessary to inhibit the enzyme.

Rates of Reaction. The rate of enzymatic reaction has been the least studied parameter of the three (onset and maximum extent being the other two) in dehydrated systems. In fact, there have been few attempts to analyze the data in a conventional kinetic manner. It has consistently been observed that the rate always increases with the quality of water in the system, the one exception being the wheat bran/olive oil system of Drapron (1972) where the decrease in rate above $a_w = 0.80$ was attributed to a thermal effect.

Many complicating factors have made kinetic analysis of published data difficult if not impossible. In particular, experimental variations in composition, control of water activity, presence of water-binding agents, and often changes in the state of substrates (e.g., crystalline vs amorphous sucrose, gelatinized vs ungelatinized starch) contribute to this difficulty.

Recently, enzyme reactions at low levels of hydration were studied by Silver and Karel (1981). They studied sucrose hydrolysis in a system containing also invertase and microcrystalline cellulose. The reaction was carried out at several temperature–water activity combinations.

An analysis of the results seemed to indicate that bulk diffusion of sucrose in this system was not the factor limiting extent of the reaction. The results seemed consistent, however, with the following model of the effects of hydration: At high moisture content, sucrose diffusion through a resistance shell confined to the vicinity of an invertase molecule is limiting. At low moisture content, water activity and temperature may affect the conformation of the enzyme itself or the diffusion coefficient in the rate-controlling resistance shell (Silver and Karel 1981).

Models of Enzyme Activity at Low Hydration. The classic studies (Acker 1962; Drapron 1977) have focused on diffusional limitations, on transport of substrate or product, or on both. And indeed, there is no doubt that water activity affects the mobility of solutes.

As mentioned previously, hydration-sensitive barriers located in the immediate vicinity of the enzyme were also a possibility mentioned for invertase by Silver and Karel (1981). In addition, a very recent paper published by a group of Chinese workers reviews some of the

theories of such protein conformation-dependent barriers (Chou and Zhou 1982).

Recent studies, however, have been quite strongly indicative of the possibility that the effect of hydration on conformation of the protein is also a major consequence in determining the effects of water activity on enzyme activity. In this respect, we wish to refer in particular to studies of Tome *et al.* (1978) who showed that in systems of low viscosity effects of water activity very similar to those observed in low water activity dehydrated systems, using aqueous solution of polar liquids. This entirely phenomenologically based interpretation of the kinetics receives strong support from a recent crystallographic study by Poole and Finney (1983). These authors used direct differential infrared and laser Raman spectroscopies to monitor sequential hydration of lysozyme. The authors note conformational changes in lysozyme as a consequence of hydration to levels lower than monolayer value. The largest conformational changes are noted at hydration levels just below the onset of enzyme activity. The authors state "it is tempting to suggest that this solvent-related effect is required before (enzyme) activity is possible." Baker *et al.* (1983) reported a hydrogen exchange study which supports the existence of hydration-related conformational changes in lysozyme.

Effects of Water Activity on Nonenzymatic Browning

Nonenzymatic browning is one of the major chemical reactions occurring in foods. The overall nature of this reaction and of the reactants and products is discussed elsewhere in this volume. This reaction has been studied extensively and several major reviews have been published.

The most sensitive products in as far as changes occurring in storage are concerned are dry and concentrated products. The major concern in processing is in heating, drying, and concentrating.

Among the environmental factors of storage that affect browning, temperature and water activity are of particular importance.

The nonenzymatic browning reaction shows a maximum at intermediate moisture contents because of water's dual role as a solvent and as a product of the reaction. At low water activity the limiting factor is inadequate mobility; therefore, addition of water, which solubilizes or plasticizes the system, promotes the reaction. At high water contents, however, the dilution of reactants and the product inhibition of condensation reactions by water, which is a condensation product, predominate, and water strongly inhibits browning. The exact position of browning maxima depends on specific products, but gen-

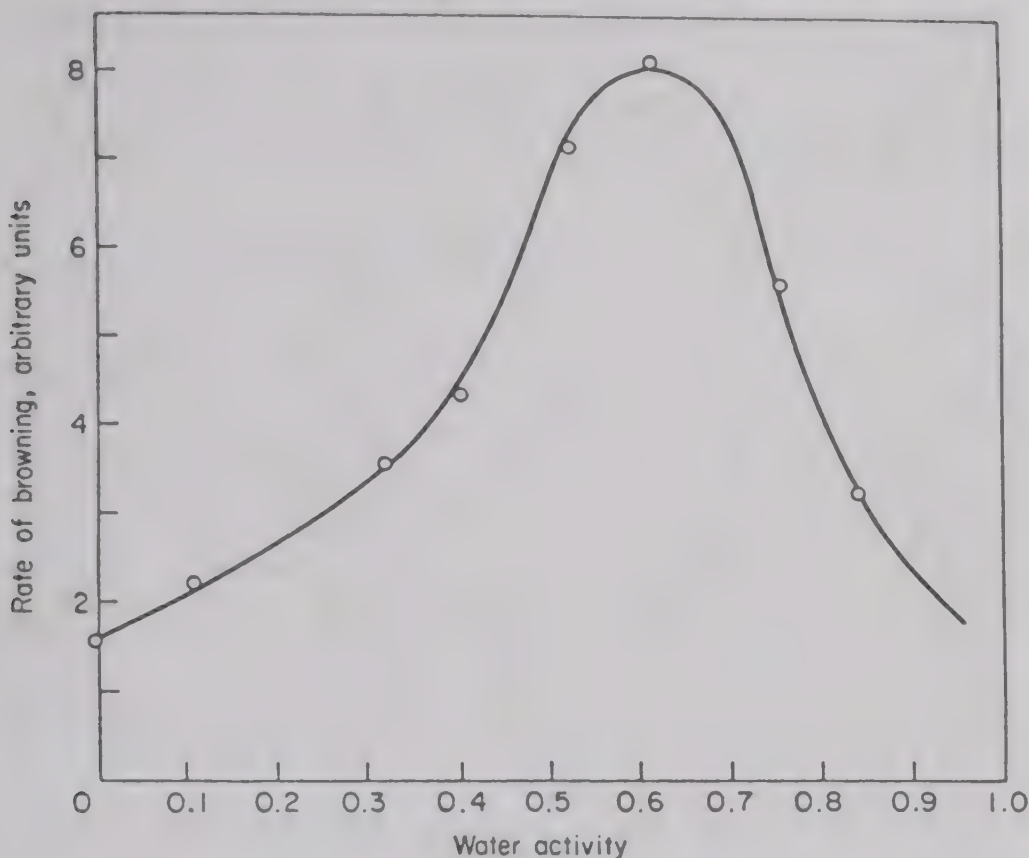


FIG. 20.1. Effect of water activity on browning of dehydrated pork.

erally, concentrated liquids (e.g., unfrozen fruit concentrates) and intermediate moisture foods (e.g., pie fillings and "evaporated" fruits such as prunes) are in the range of moisture content most susceptible to browning.

As indicated, in low moisture foods such as dehydrated fruit, vegetables, milk, eggs, and meat products, the browning rate increases with increasing moisture content up to a maximum. Typical maximum behavior is shown in Fig. 20.1, which gives data for a dehydrated pork product used in space feeding. Water content, however, affects not only the rate at a given temperature, but also the activation energy for browning. Figure 20.2 shows the activation energy as a function of moisture for the case of dehydrated cabbage.

Very often the kinetic equations describing nonenzymatic browning in foods are complex and may require computer-aided simulation for prediction of their shelf life. Such examples have been described in the work of Mizrahi *et al.* (1970) and Mishkin *et al.* (1983A).

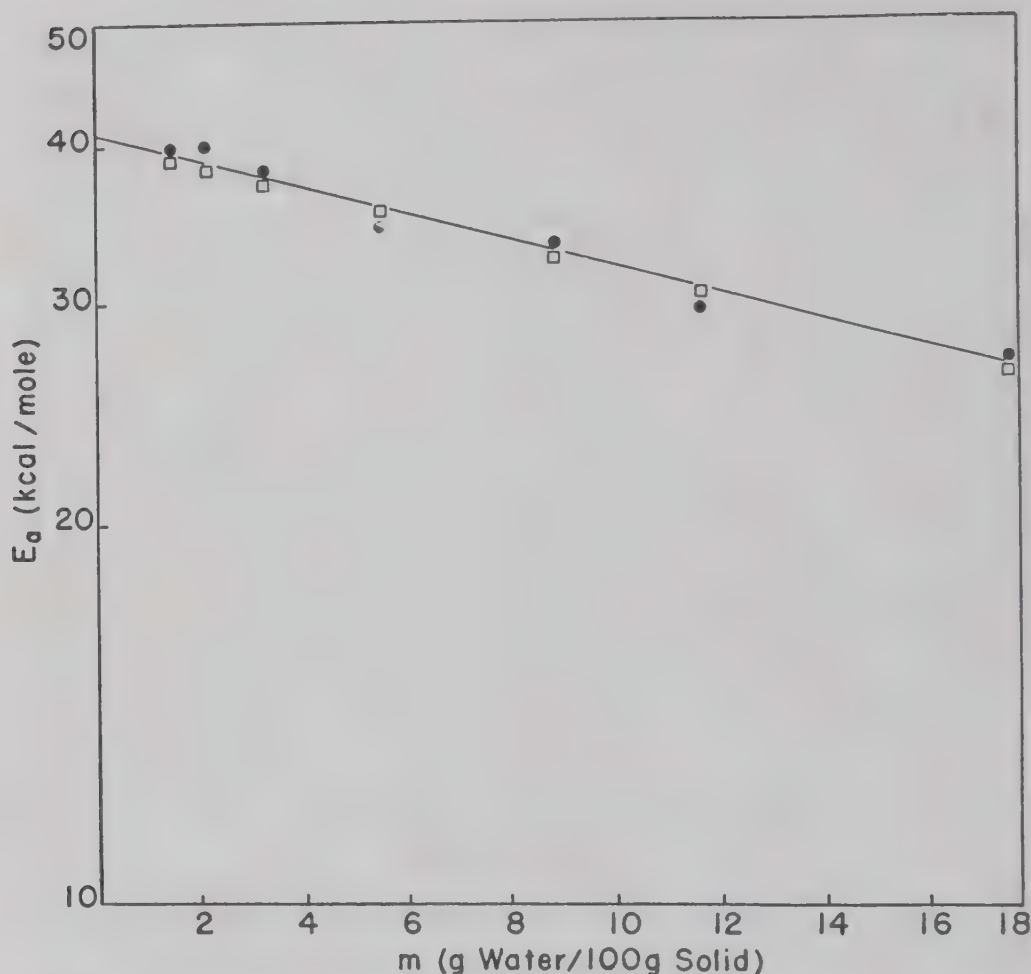


FIG. 20.2. Effect of water activity on activation energy for browning in cabbage. ●, Experiment points; □, extrapolated from three highest moisture contents.

On the other extreme are very simple approximations to the effects of moisture and of temperature on the browning reaction in foods. For low moisture vegetables it was possible to correlate extensive data in the literature on discoloration with the moisture content and temperature by a very simple equation (Villota *et al.* 1980):

$$\ln t_f = a_0 + a_1(1/T) + a_2(m - m_1) \quad (5)$$

where t_f the time of failure (days), T the absolute temperature (°K), m the moisture content (g H₂O/g total weight), m_1 the BET monolayer moisture, and a_0 , a_1 , a_2 are constants.

In the case of vegetables stored under N₂ in which color changes

were due to nonenzymatic browning and the color changes were limiting shelf life, values of a_0 ranged from (-52) to (-35) , of a_1 from (14.4×10^3) to (17.8×10^3) , and of a_2 from (-0.536) to (-0.139) . The values of a_1 were consistent with activation energies of 29 to 35 kcal/mol.

Effects of Water Activity on Oxidative Reactions in Foods

The major oxidative reactions in foods are due to peroxidation of lipids and of some water-soluble vitamins. Major products of oxidation of lipids, such as fatty acids, acylglycerols, and phospholipids are the peroxides of these lipids. These peroxides can then react with the other food constituents, or they may decompose to secondary products, such as aldehydes, ketones, hydroxyacids, and hydrocarbons, which are often volatile and may have strong odor and off-flavor. It is these smaller, volatile compounds that cause rancidity of foods. These secondary products as well as peroxides and lipid free radicals can react with proteins and vitamins, causing losses in nutritional value and in solubility of food constituents.

The major factors that affect oxidation include the presence of catalysts and antioxidants, light wavelength and intensity, oxygen pressure, water activity, and temperature. Increasing the temperature does increase the rate of free-radical reactions, but the activation energy is lower than in the case of nonenzymatic browning and is usually below 20 kcal/mol.

In the case of oxidation of water-soluble vitamins, exemplified by ascorbic acid, the activation energy is dependent on reaction conditions (Archer and Tannenbaum, 1979). Oxygen dependence is discussed in a subsequent section, but it should be noted that it is in part modified by the level of water activity. Thus, in low pH, dehydrated fruit, and vegetable powders, the ascorbic acid degradation is very strongly dependent on water activity, but not on oxygen concentration. Dilute systems of similar composition, however, which have a high water activity show a pronounced oxygen dependence. The oxidation of ascorbic acid in the low water activity systems is extremely dependent on water activity. A number of studies have been conducted on this topic and they agree in this respect. The dependence is often exponential with the logarithm of the rate constant proportional to water activity, but other relations including polynomial function have also been used to relate rate to water activity (Labuza 1972; Riemer and Karel 1978; Villota and Karel 1980; Mishkin 1983).

Lipid oxidation in foods is associated almost exclusively with un-

saturated fatty acids and is often autocatalytic, with oxidation products themselves catalyzing the reaction so that the rate increases with time. The reaction may also be catalyzed by metals or the enzyme lipoxygenase. Both the catalyzed and autocatalytic reactions are initiated by decomposition of hydroperoxides to free radicals. In pure systems, monomolecular decomposition occurs up to at least 1–2% oxidation (molar basis), with the rate and time course proportional to the square root of the extent of oxidation. During this period all oxygen absorbed forms hydroperoxides. Above 1% oxidation, bimolecular decomposition of hydroperoxides becomes controlling, the rate being proportional to the peroxide concentration.

Water activity is a major factor affecting the rate of lipid oxidation in foods. It has long been recognized that very low water contents in fat-containing foods are conducive to rapid oxidation, and the literature documenting this phenomenon is voluminous (Karel and Yong 1981).

Several hypotheses have been advanced to explain water's retarding effect on lipid oxidation. One factor that may be important is production of antioxidants through nonenzymatic browning. The effectiveness of products of nonenzymatic browning was demonstrated early by Griffith and Johnson (1957) and was one of the mechanisms hypothesized by Karel (1960). Several investigators in Japan and Europe have confirmed recently that intermediates in the complex set of reactions we term nonenzymatic browning are effective antioxidants. Furthermore, intermediate moisture contents would maximize the concentration of these intermediates (Eichner 1981). Since high water activity promotes browning, this may be one of the explanations for the observed effects.

Studies on purified model systems, containing no components capable of forming antioxidants through browning, however, also show retardation by increasing water content, and the explanation for these effects is probably based on the effect of water on initiation and termination steps of the chain reaction.

In purified systems, water interferes with the normal bimolecular decomposition of hydroperoxides by hydrogen bonding with amphipolar hydroperoxides formed at the lipid–water interface. In the presence of trace metals added to model systems of lyophilized emulsions, humidification at moderate levels retards oxidation because of hydration of metal ions. The reduction in rate depends on the type and hydration state of the metal salt added as well as on water content. The monomolecular rate period is primarily affected by this mechanism, although bimolecular rates are also decreased (Ksrel *et al.* 1967).

Relatively less is known about water activity's effect on termina-

tion, but it is to be expected that water would increase mobility and hence promote recombination of free radicals.

Water may also be expected to influence free-radical interactions between oxidizing lipids and other food components by influencing the concentrations of initiating radicals present, the degree of contact and mobility of reactants, and the relative importance of radical transfer recombination reactions. In particular, the reactions between peroxidizing lipids and proteins are strongly affected by water activity. Increasing water activity promotes the formation of Schiff base type of cross-links between proteins, with bifunctional fragments of oxidized lipids incorporated as cross-linking agents. On the other hand, at low water activities free-radical cross-links predominate, and the proteins cross-link without the incorporation of lipid fragments (Funes and Karel 1981).

Retardation of peroxidation by increasing water content is reversed at high water activity. As water contents are increased above monolayer coverage in foods and in model systems, resistance to diffusion of solutes decreases and solubilization becomes significant. In systems containing chelating agents and antioxidants, high water contents allow solubilization of chelating agents that sequester metals as well as solubilization of antioxidants. This effect lowers the oxidation rate. However, at high water activities water may accelerate oxidation by solubilizing catalysts or by inducing swelling macromolecules such as proteins to expose additional catalytic sites.

A little-explored aspect is water's effect on food structure and on oxygen's ability to reach oxidation-susceptible food components. This aspect has been investigated thoroughly in recent years by Flink and co-workers (Karel and Flink 1983). They studied the influence of physical structure of freeze-dried emulsified systems on the oxidation behavior of the lipid component. The partitioning of lipid into encapsulated and surface fractions was evaluated microscopically and chemically in emulsion systems of varying composition and concentrations prepared under selected processing conditions and drying behavior. These studies demonstrated the influence of structure formation and lipid partitioning on subsequent oxidation behavior.

In systems in which there are both surface lipids and lipids encapsulated within a carbohydrate, polysaccharide, or protein matrix, the surface lipids oxidize readily when exposed to air. The encapsulated lipids, however, do not oxidize until the structure of the encapsulated matrix is modified or destroyed by absorption of water. Prevention of this structure is of considerable importance in protecting unsaturated lipids from oxidation in dry preparation.

Effect of Oxygen Concentration on Oxidation of Lipids in Foods

When the effect of oxygen on oxidation of lipids in processing is considered, it is often simply a question of total amount available for reaction with food components. If this amount is limited to a level that causes no significant effect in the food and there is no potential for additional oxygen coming into contact with the food, then the rate of reaction is irrelevant. In other cases, the total amount of oxygen potentially able to react with food components is, in fact, significant; and consideration must be given to the effect of concentration (or partial pressure) of oxygen on the rate.

The oxidation of unsaturated lipids is a free radical reaction with an initiation step, a propagation step, and a termination step. The

TABLE 20.3. FACTORS CONTROLLING RATE OF LIPID OXIDATION^a

Initiation
Hydroperoxide concentration
Presence of catalysts
Electromagnetic radiation (includes light)
Chemical initiators (includes some oxidation products)
Propagation
Degree of unsaturation
Local oxygen concentration
Termination and branching
Antioxidant concentration

^a Temperature and water activity are factors in all of above steps.

rate is strongly affected by environmental and compositional factors, and these are summarized in Table 20.3. As indicated in this figure, all of the steps of the reaction are affected by temperature and by water activity, which were discussed previously.

Kinetics of free radical reactions are complicated, but can be greatly simplified by using simplifying assumptions which allow the prediction of trends that reactions will follow, but may be insufficiently accurate to predict quantitatively the exact extent of the reaction. Such greatly simplified equations are shown in Table 20.4

These equations show how important it is to minimize the rate of initiation of free radicals. No matter what the other conditions are, the rate of oxidation increases with the rate of initiation, and the antioxidant concentration remaining in the system is also depleted at a rate that is equal to the rate of initiation. Therefore, if the rate of

TABLE 20.4. GREATLY SIMPLIFIED OXIDATION KINETICS^a

No. antioxidant, high O ₂ pressure
$R_o = K_1 (RH)(R_i)^{1/2}$
No antioxidant, low O ₂ pressure
$R_o = K_2 (O_2)(R_i)^{1/2}$
Antioxidant, low O ₂ pressure
$R_o = K_3 (O_2)(AH)^{-1}(R_i)$
Rate of loss of antioxidant = R_A
$R_A = R_i$

^a R_o = rate of oxygen absorption;
 R_i = rate of initiation.

initiation is high, then even the addition of antioxidants is only marginally effective in retarding oxidation.

A general form often found useful in correlating reaction rate with oxygen concentration is given as

$$R = \frac{[O_2]}{k_1 + k_2[O_2]} \quad (6)$$

where R is the reaction rate, $[O_2]$ the oxygen concentration, and k_1 , k_2 are constants.

Note that when $k_2[O_2]$ is much smaller than k_1 , the rate is linear with oxygen concentration, and when $k_2[O_2]$ is much larger than k_1 , the rate becomes independent of concentration. A general shape of the oxygen dependence is shown in Fig. 20.3. It should be evident that in order to avoid oxidation, very low oxygen concentrations are necessary.

Effects of Oxygen on Color Changes in Prepackaged Meats

The consumer is greatly influenced by the color of meat at the time of purchase. In spite of the fact that in modern technology the traditional relation between loss of bright red color and age of meat no longer exists, and in spite of the fact that most meats are consumed after cooking (which changes the color), the consumer wishes to purchase meats that have a bright red color, and also wishes to have transparent packages in which this color can be observed. These considerations complicate the optimization of meat quality. The color of meats is due to the protein pigment myoglobin. In the presence of oxygen this pigment forms the oxygenated oxymyoglobin. Both of these

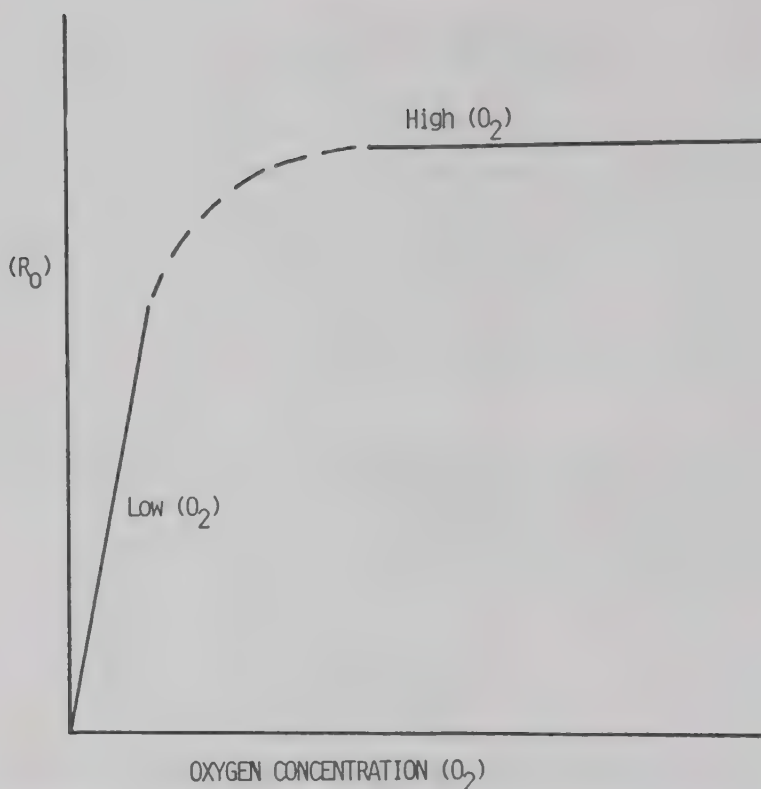


FIG. 20.3. Effect of oxygen concentration on rate of oxidation.

pigments contain heme iron in the reduced (ferrous) state. Upon oxidation of the iron, the pigment becomes the brown metmyoglobin. On heating this pigment is denatured. The reactions involved are complex and have been recently reviewed in detail by Livingston and Brown (1981).

The desirable color of fresh meats is due to oxygenated ferrous myoglobin and in cured meats to nitrosomyoglobin. Both pigments can oxidize to ferric myoglobin forms which are much less desirable, and the dependence of this oxidation on oxygen concentration is complex (Karel 1974). Processing by ionizing radiation which is a potential pasteurization or sterilization process for meats further complicates this oxygen dependence (Kamarei *et al.* 1981).

OPTIMIZATION OF FOOD PROCESSES ON THE BASIS OF KINETIC MODELS RELATING PROCESS VARIABLE TO CHEMICAL CHANGES

Once a kinetic model is available that relates the rate of change of a quality index to environmental factors which may be controlled in

a process, it is feasible to simulate the changes in that quality index as a function of process conditions. More significantly, it is possible then to optimize the process to achieve the best quality attainable within a set of constraints imposed by physical, economic, or biological aspects of the process.

Examples of such optimization are still limited in the literature of food science and technology, but their number is increasing and the complexity of problems tackled is increasing as well. Recently, optimization studies were undertaken at The Massachusetts Institute of Technology with respect to problems of sterilization and dehydration. Saguy and Karel (1979) calculated the optimal retort temperature profile for maximizing thiamine retention during sterilization of a typical food in three different can sizes. The organism to be sterilized was assumed to have the thermal resistance characteristics of *Bacillus stearothermophilus*. They showed that a single optimal profile exists, giving, however, only a slight improvement over constant retort temperature processes.

More recently, Mishkin *et al.* (1982) published results of their studies on optimization of air dehydration processes. A variety of constraints on time and maximum process temperature were made and optimal dryer temperature profiles were established for the following:

1. Minimizing browning in drying of potatoes (Mishkin *et al.* 1983B),
2. Maximizing ascorbic acid retention in drying of potatoes (Mishkin *et al.* 1982; Mishkin 1983), and
3. Maximizing ascorbic acid loss, with a constraint on enzyme inactivation in a food model (Mishkin *et al.* 1983B).

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